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## Introduction

Progestins and glucocorticoids are two classes of steroid hormones with very distinct biological functions. The major physiological role of progestins in the mammal are to establish and maintain pregnancy in the uterus and ovary; to promote lobular-alveolar development of the mammary gland and to suppress milk protein synthesis during pregnancy (1). Some classical biological actions of glucocorticoids are regulation of metabolism, suppression of bone formation, and inhibition of inflammation and the immune system (2). In the pathology of breast cancer, it has been shown that progestin agonist can increase the incidence of spontaneous mammary tumors. Some studies indicated that physiological doses of progestin agonists can stimulate the growth of established tumors, while high pharmacological dose progestins can inhibit the growth of established tumors (3). However, there is no evidence of a role of glucocorticoids in breast cancer even though both progesterone receptor and glucocorticoid receptor are expressed in the mammary gland. Interestingly, progesterone receptor (PR) and glucocorticoid receptor (GR) share many similar structural and functional characteristics: similar amino acid sequences and functional domains; similar, if not identical, DNA sequence recognition specificity; associate with a similar complex of molecular chaperones in the absence of hormone and with a similar set of coactivators or corepressors in the presence of hormone. How can two receptors with such remarkable similarity mediate such dramatically different biological functions? We hypothesize that one way that progestins and glucocorticoids can exert different biological effects is through their different abilities to regulate the expression of certain target genes. In order to understand the role of progestins in breast cancer and how progesterone receptor mediates this hormone specific regulation, we have identified genes that are differentially regulated by progestins and glucocorticoids using two systematic strategies: a retroviral promoter-trapping strategy and an Affymetrix oligonucleotide microarray analysis. The long-term goal of this investigation is to understand the mechanisms of the differential hormone regulation. The genes identified in this investigation play an important role in hormone functional specificity. Knowledge gained from this investigation will be fundamental to the understanding of both hormone actions and their roles in breast cancer.



## Body

**AIM ONE:** Develop a retroviral promoter-trapping strategy to identify promoters/enhancers differentially induced by progestins and glucocorticoids. (Detailed description and all figures are included in Appendix A.)

### Establishment of a Cre/*loxP* mediated retroviral promoter-trapping system

To obtain a cell line that can sensitively detect Cre recombinase expression, the multifunctional reporter plasmid (ppgklxtkneo/hygro) (Fig. 1) was stably transfected into 4F fibroblasts that express both GR and PR. This reporter plasmid consists of two, tandemly arrayed selective marker genes that are transcribed from a *pgk* promoter. The 5' marker encodes for a fusion protein between herpes simplex virus 2 thymidine kinase (*tk*) and the neomycin phosphotransferase (*neo*), and is flanked by two direct-repeats of the *loxP* recombination target site (4). The 3' marker encodes for hygromycin phosphotransferase (*hygro*) and is located downstream of the second *loxP* site. In the absence of Cre, the *tkneo* gene is expressed from the constitutive *pgk* promoter. The *hygro* gene does not have a promoter immediately upstream and chimeric transcripts initiated at the *pgk* promoter are suppressed by two tandem copies of the bovine growth hormone polyadenylation sequence upstream of *hygro*. Therefore, the cells containing a single copy of ppgklxtkneo/hygro are G418-resistant but hygromycin-sensitive. However, when Cre is expressed, it will catalyze recombination between the two *loxP* sites resulting in the deletion of *tkneo*. This places *hygro* immediately downstream of the *pgk* promoter rendering the cells G418-sensitive but hygromycin-resistant. Thus, 4F cells with a stably integrated ppgklxtkneo/hygro selection plasmid report even transient expression of Cre by undergoing a permanent switch in their drug-resistance phenotype. A G418-resistant clone (4Fneo/hygro21) containing a single copy plasmid and could undergo a clean drug-resistance phenotype switch after Cre expression was selected (see Appendix A).

In this gene-trapping system, we employed a self-inactivating retroviral gene-trap vector (U3Cre) in which a promoterless Cre gene is inserted in the U3 region of an enhancer-deleted long terminal repeat (Fig. 2). Viral replication and long terminal repeat-mediated duplication place the promoter-less Cre sequence just 30 nucleotides downstream of the flanking cellular DNA (4-6). Therefore, Cre is not expressed unless the virus integrates into an active cellular promoter. In this work, we employ this feature to identify hormone-regulated promoters.

### Isolation of cell lines representing trapped, hormone inducible and differentially hormone inducible promoters

The U3Cre plasmid was stably transfected into an amphotropic packaging cell line PA317 (7). Supernatants from lines producing high titers of recombinant virus were used to infect 4Fneo/hygro21 cells (5,6) resulting in a library consisting of approximately  $4 \times 10^6$  independent proviral integration events. Clonal cell lines were isolated following sequential drug selections as outlined in Fig. 3 in Appendix A. The selection scheme for cell clones of group A enriches for promoters induced by progestins whereas group B is enriched for promoters induced by glucocorticoids. Trapped promoters preferentially induced by glucocorticoids vs. progestins are enriched in cell clones of group C and conversely promoters preferentially induced by progestins are trapped in group D. Forty cell lines from each of group A and group B, and an additional twenty cell lines from each of group C and group D were isolated for further analysis.

To determine the expression pattern of the gene represented in each clonal cell line isolated, we developed a reporter assay to quantitate the Cre protein level based on Cre recombinase activity (Fig. 4). A reporter plasmid (ppgklxtkneo/luc) was constructed in which the *hygro* gene of the multifunctional selection plasmid was replaced by luciferase (*luc*). Luciferase expression from this plasmid was dependent on Cre expression. A titration of Cre expression vector demonstrated that the higher the input of Cre, the higher the output of luciferase expression. Therefore, this sensitive and quantitative luciferase reporter strategy can determine the exact regulation pattern of the trapped promoters in each isolated clones and identify the lines representing differentially regulated genes.

Each clonal cell line with potentially hormone-regulated, trapped promoters was transfected with ppgklxtkneo/luc and treated with dexamethasone or R5020 24 hrs later. The induction of Cre activity was determined by measuring luciferase activity. In 58 cell lines, luciferase expression was induced by either glucocorticoids or progestins by more than 2 fold. In 19 cell lines, luciferase expression was differentially regulated by the two hormones by more than 1.5 fold (Fig. 4C).

### **Hormone regulation of selected promoters**

In order to determine whether the endogenous cellular genes trapped in the clonal cell lines are indeed hormone regulated, genomic DNA sequences upstream of the proviral integration site of several clonal isolates were retrieved by inverse PCR and sequenced (Fig. 5) (4,5). Blast searches using different databases revealed that the entire sequence upstream of the proviral integration site in clone 43 (441bp) is 98% identical with the expressed sequence tag (EST) ic84f11.x1 in the cDNA library "Melton Normalized Mixed Mouse Pancreas 1 N1-MMS1" (dbEST ID 9280663, GenBank accession number BI438077). However, the sequences upstream of the proviral integration sites in clone 32, 42 and 66 showed no significant homology to known genes or expressed sequence tags at the time of analysis. These sequences have been deposited in GenBank with accession numbers AF465703 (clone 32 gene), AF465704 (clone 42 gene) and AF465705 (clone 66 gene).

The mRNA level of the gene represented by clone 32 (clone 32 gene) in the parental cell line (4F) was measured by semi-quantitative RT-PCR (Fig. 6). Clone 32 gene was preferentially induced by glucocorticoids (5 fold) than by progestins (2.5 fold). Although this is only a two-fold difference in the induction, it is more impressive when one considers that progesterone receptors are 10-fold more abundant than glucocorticoid receptors in these cells. Expressions of the genes represented by the other two clones were quantitated in the same way (Fig. 7). Clone 43 gene was also preferentially induced by glucocorticoids. Clone 42 gene was induced by both hormones to a similar level. For all three clones, the mRNA regulation patterns of the endogenous genes agree with the Cre protein regulation patterns shown in Figure 4C, suggesting that the genes identified here are indeed regulated by hormones.

### **Glucocorticoid induction of clone 32 gene is dependent on GR and does not require *de novo* protein synthesis**

A dose-response curve demonstrated that even at concentration as low as 1nM, dexamethasone can induce clone 32 gene (Fig. 9A), suggesting that the induction of clone 32 gene by glucocorticoid is specific. The induction of clone 32 gene by either dexamethasone or R5020 was completely abolished following antagonist RU486 treatment. In contrast, the induction by R5020 is abolished while induction by dexamethasone is only slightly reduced following ZK112 treatment

(Fig. 9B), indicating that the glucocorticoid induction of clone 32 gene is GR-dependent. Furthermore, the induction of clone 32 gene by dexamethasone was maintained following cycloheximide treatment (Fig. 9C), suggesting that the glucocorticoid induction is a direct response that does not require *de novo* protein synthesis.

### **Glucocorticoid induction of clone 32 gene is an early response and is cell-type specific**

A time course study demonstrated that the glucocorticoid induction of clone 32 gene peaked as early as 30 min (Fig. 10A), again indicating this is a direct response of hormone regulation. The hormone induction lasted for at least 22 hrs. In addition, clone 32 gene was induced by glucocorticoids in another mouse fibroblast-derived L929 cells, but not in the human mammary carcinoma-derived T47D/A1-2 cells (Fig. 10B), indicating that the hormone regulation of clone 32 gene is cell-type specific.

Despite the disparate biological activities of glucocorticoids and progestins, there is little information on what genes may be differentially regulated by the two hormones and how this may be accomplished. Our studies document a novel approach toward addressing this void. Because Cre-mediated recombination is a permanent event, the expression of the selectable marker genes is independent of ongoing Cre expression. Thus this system permits the identification of genes that are transiently or weakly induced by hormone. Further studies on the mechanisms underlying the differential regulation will enhance our understanding of how glucocorticoids and progestins function as specific physiological regulators or therapeutic agents.

**AIM TWO:** Implement Affymetrix oligonucleotide array analysis to identify genes differentially regulated by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2. **(Detailed description and all figures are included in Appendix B.)**

### **Overlapping but distinct gene regulation profiles by glucocorticoids and progestins**

In this study, we performed Affymetrix microarray analysis of gene expression in the human breast cancer cell line T47D/A1-2 that express comparable levels of both GR and PR (8).

Total RNA was isolated from cells treated with vehicle, dexamethasone (Dex, 100 nM) or R5020 (10 nM) for 2 hrs or 6 hrs. Probes generated from this RNA were hybridized to Affymetrix HuGeneFL Arrays to analyze expression of 5,600 full-length human genes.

Of 5,600 genes analyzed, 70 were upregulated by glucocorticoids by more than 3-fold at either 2hr or 6hr treatment, and another 33 were downregulated by more than 3-fold. The number of genes that are upregulated and downregulated by progestins by more than 3-fold are 47 and 34, respectively. The majority of the genes (>90%) identified have not been previously described to be regulated by either glucocorticoids or progestins and therefore represent novel hormone-regulated targets. Among these genes, 25 were upregulated by both hormones (Table 1) and 12 were downregulated by both hormones by more than 3-fold (Table 2). Of particular interest for this work, 31 genes have been identified to be differentially regulated by the two hormones by more than 3-fold (Table 3). These results demonstrate that glucocorticoids and progestins regulate overlapping but distinct sets of genes. In light of the paucity of genes described to be differentially regulated by the two hormones, it was surprising that the number of genes differentially regulated by more than 3-fold, 31, approached the number regulated by both by more than 3-fold, 37. These represented about 1.5-2% of the genes whose expression could be detected.

### **Confirmation of differential regulation with RT-PCR and northern blot analyses**

The expression of four genes as representing different patterns of regulation was confirmed by both semi-quantitative RT-PCR and northern blot (Fig. 2A). 1) G0S8 (also known as RGS2, Regulator of G protein Signaling 2) is specifically induced by Dex but not by R5020 (Fig. 2A). Quantitation of the northern blot showed that Dex treatment led to a more than 20-fold induction while R5020 treatment had minimal effect. 2) Like G0S8/RGS2, PLZF (promyelocytic leukemia zinc finger protein) is strongly induced by Dex (Fig. 2B) but unlike G0S8/RGS2, PLZF is also weakly induced by R5020. In addition, northern blot showed that PLZF is expressed as two transcripts, 11 kb and 9 kb. Both transcripts are induced by Dex at three times higher fold than by R5020. 3) In contrast to PLZF and G0S8/RGS2, the gene for the  $\beta$ B subunit of inhibins and activins (INHBB for inhibin  $\beta$ B) is specifically induced by progestins but not glucocorticoids (Fig. 2C). Northern blot showed that there are two transcripts for INHBB as seen previously for rat INHBB (9). R5020 treatment induced the expression of both transcripts, while Dex treatment had no effect on the 4 kb transcript and slightly down-regulated the 3 kb transcript. 4) Unlike the previous three genes, the differential regulation of the IEX-1/Dif-2 gene involves a hormone-specific downregulation. Northern blot demonstrated that Dex treatment led to a dramatic 80% down-regulation by 6 hr, whereas R5020 treatment resulted in a weak, transient down-regulation with expression returning to control levels by 6 hr (Fig. 2D).

### **Hormone regulation is direct and receptor-mediated**

To determine whether the differential regulation of the four genes above was mediated by the cognate receptors and direct, T47D/A1-2 cells were treated with protein synthesis inhibitor cycloheximide or the GR/PR antagonist RU486 along with Dex or R5020 (Fig. 3). Hormone regulation pattern is maintained following cycloheximide treatment, indicating that the hormone regulation is a direct effect that does not require *de novo* protein synthesis. In contrast, the hormone regulation is completely abolished following RU486 treatment, indicating that hormonal regulation is mediated through GR and/or PR.

### **Differential gene regulation by glucocorticoids and progestins mediate hormone specific effects**

Progestins are associated with the incidence and progression of breast cancer (3). Recent work suggests that progestins may prime mammary cells to respond to growth factors (10). In contrast, glucocorticoids are growth suppressive in mammary cancer cells (11,12). To test whether glucocorticoids and progestins have different effects on the proliferation of T47D/A1-2 cells, we assessed cell growth by monitoring the total DNA content of hormone treated cell populations (Fig. 4A), and cell cycle progression by flow cytometry analysis of propidium iodide stained cells (Fig. 4B). In summary, these studies clearly demonstrated that during the first 24 hrs of hormone treatment, glucocorticoids are growth suppressive, while progestins are growth stimulating. The early time points (2 hr and 6 hr) used in our microarray analysis allowed us to assess the early molecular changes occurred that may account for this differential growth effects of the two hormone on the T47D/A1-2 cells.

From the examination of the differentially regulated genes identified, a pattern emerged that gives insight into the differential effects of the two hormones on cell proliferation. A number of the genes that are preferentially induced by glucocorticoids or suppressed by progestins are growth suppressive (Table 3, top). G0S8/RGS2 has been shown to be induced in growth-arrested cells and to promote adipocyte differentiation (13). PLZF has been shown to



suppress the growth of myeloid cells by inducing G0/G1 arrest and apoptosis, partly through the binding and repression of the cyclin A2 promoter (14,15). RAR $\gamma$  inhibits proliferation and activates apoptosis in breast cancer cells (16,17). VDUP1 (Vitamin D3 Up-regulated Protein 1) suppresses cell proliferation by inhibiting the reducing potential of the disulfide reducing protein thioredoxin and down-regulating thioredoxin expression (18-20). Expression of VDUP1 is down-regulated in chemically-induced rat mammary tumors (21). Thus, in mammary cancer cells, the growth-suppressive effect of glucocorticoids may be mediated through the induction of G0S8/RGS2 and PLZF, while the growth-promoting effect of progestins may be mediated through the down-regulation of RAR $\gamma$  and VDUP1.

In contrast, many of the genes that are preferentially induced by progestins or suppressed by glucocorticoids are potentially proliferation-related (Table 3, bottom). Mac-2 Binding Protein/90K is a tumor-derived antigen and is expressed at elevated levels in the serum of patients with breast cancer and other types of cancer (22). Alpha-N-acetylgalactosaminidase (NaGalase) is an extracellular matrix-degrading enzyme that is produced exclusively by cancer cells (23). The NaGalase levels in mice bearing squamous cell carcinoma increased with time of tumor growth and were directly proportional to tumor burden (24). Inhibin  $\beta$ B promotes mammary gland ductal elongation and alveolar morphogenesis (25). MAP kinase phosphatase (MKP-2) can be induced by growth factors and is up-regulated in cells transformed by v-Jun or mutated K-ras (26,27). Pim-2 is a protooncogene that induces lymphoid tumors synergistically with c-myc in mice (28,29). IEX-1/Dif-2 gene can be induced by serum or growth factors (30) and is expressed in proliferating monocytes but significantly down-regulated during differentiation (31). In mammary cancer cells, growth-suppressive effect of glucocorticoids may be mediated through the down-regulation of alpha-N-acetylgalactosaminidase, MKP-2 and IEX-1/Dif-2, while growth-promoting effect of progestins may be mediated through the induction of Mac-2 BP/90K, INHBB and Pim-2h.

In addition to the effects on mammary cancer cell growth, glucocorticoids promote milk protein synthesis and lactation (32-34), while progestins inhibit milk production and secretion (1). VIP (vasoactive intestinal polypeptide) receptor related protein is the long isoform of VIP receptor. VIP has been shown to be a physiological mediator of prolactin release in the rat (35). It is likely that glucocorticoid-specific stimulation of lactation is, in part, mediated by glucocorticoid-specific induction of VIP receptors (Table 3).

In summary, the genes identified to be differentially regulated by glucocorticoids and progestins provide potential mechanisms through which the two hormones exert different or opposite biological effects. With a better understanding of the functionally uncharacterized genes identified here, more potential mechanisms will emerge. Future studies exploring these potential mechanisms will assist in developing tumor markers and therapeutic agents for cancer. In addition, genes identified in this study can be used as model systems to investigate the molecular mechanisms underlying differential gene regulation by glucocorticoids and progestins. The fruits of these investigations will enhance our appreciation of the larger question of how related transcription factors mediate distinct, even opposing, biological actions.

## Key Research Accomplishments

### Aim One:

- A Cre/*loxP* mediated retroviral promoter-trapping system has been established.
- Using sequential drug selection, 58 cell lines have been isolated that represent potential promoters inducible by either glucocorticoids or progestins by more than 2 fold. Another 19 cell lines have been isolated that represent promoters potentially differentially regulated by the two hormones by more than 1.5 fold.
- By assessing the mRNA level of the endogenous genes identified, two novel genes have been confirmed to be differentially regulated by glucocorticoids and progestins. Another novel gene has been confirmed to be induced by both hormones.
- Glucocorticoid induction of clone 32 gene has been shown to be GR dependent, *de novo* protein synthesis independent, an early response and cell-type specific.

### Aim Two:

- In the Affymetrix microarray analysis, of 5600 genes analyzed, 70 are upregulated by glucocorticoids by more than 3-fold at either 2hr or 6hr treatment, and another 33 are downregulated by more than 3-fold. The number of genes that are upregulated and downregulated by progestins by more than 3-fold are 47 and 34, respectively. Among these genes, 25 are upregulated by both hormones and 12 are downregulated by both hormones by more than 3-fold. Of particular interest for this work, 31 genes have been identified to be differentially regulated by the two hormones by more than 3-fold.
- The expression of four genes as representing different patterns of regulation has been confirmed by both semi-quantitative RT-PCR and northern blot, demonstrating the reliability of the array results.
- Treatment with a protein synthesis inhibitor or receptor antagonist indicated that the hormone regulation of the genes identified is protein synthesis independent but receptor dependent.
- Cell growth study and cell cycle analysis have demonstrated that during the first 24 hrs of hormone treatment, glucocorticoids are growth suppressive, while progestins are growth stimulating in T47D/A1-2 cells.
- The genes identified to be differentially hormone regulated provide potential mechanisms underlying the differential effects of the two hormones on breast cancer cell proliferation.

## Reportable Outcomes

- Wan Y and Nordeen SK (2001) Microarray analysis of differential gene regulation by glucocorticoids and progestins in human breast cancer cells. Oral presentation, 83<sup>rd</sup> Annual Meeting of The Endocrine Society, Denver, CO
- Wan Y and Nordeen SK (2002) Differential Gene Regulation by Glucocorticoids and Progestins. Poster presentation, 2002 Keystone Symposia A6 meeting "Chromatin Structure and Activity", Santa Fe, NM
- Wan Y and Nordeen SK (2002) Differential Gene Regulation by Glucocorticoids and Progestins. Poster presentation, 2002 Keystone Symposia D4 meeting "Nuclear Receptor Superfamily", Snowbird, UT
- Wan Y and Nordeen SN (2002) Overlapping but distinct gene regulation profiles by glucocorticoids and progestins in human breast cancer cells. *Molecular Endocrinology* 16(6):1204-1214
- Wan Y and Nordeen SN (2002) Identification of genes differentially regulated by glucocorticoids and progestins using a Cre/*loxP* mediated retroviral promoter-trapping strategy. *Journal of Molecular Endocrinology* 28(3):177-92
- Wan Y and Nordeen SN (submitted) Overlapping but distinct profiles of gene expression elicited by glucocorticoids and progestins. *Recent Progress in Hormone Research*.
- A database of genes that are regulated by progestins or glucocorticoids in T47D/A1-2 cells have been generated based on the result of Affymetrix HuGeneFL oligonucleotide array analysis.
- Ph.D. degree in Biophysics and Genetics from University of Colorado Health Sciences Center has been obtained by Yihong Wan on December 14, 2001 based on the research supported by this award.
- A postdoctoral fellow position in Dr. Ronald M. Evans' laboratory at The Salk Institute for Biological Studies (La Jolla, CA) has been received by Yihong Wan based on experience/training supported by this award.

## Conclusions

Despite the disparate biological activities of glucocorticoids and progestins, there is little information on what genes may be differentially regulated by the two hormones and how this may be accomplished. Our studies document two novel approaches toward addressing this void. In the Cre/Lox-mediated retroviral promoter trapping system, two novel genes have been identified to be differentially regulated by glucocorticoids and progestins, another novel gene has been found to be regulated by both hormones. In the Affymetrix microarray analysis, 31 genes have been determined to be differentially regulated by the two hormones by more than 3 fold. The set of differentially regulated genes suggests potential mechanisms underlying the distinct effects of the two hormones on breast cancer cell proliferation. Future studies exploring these potential mechanisms will assist in developing tumor markers and therapeutic agents for cancer. In addition, genes identified in this study can be used as model systems to investigate the molecular mechanisms underlying differential gene regulation by glucocorticoids and progestins. The fruits of these investigations will enhance our appreciation of the larger question of how related transcription factors mediate distinct, even opposing, biological actions.

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## **Appendices:**

### **Appendix A:** A copy of the published article

Wan Y and Nordeen SN (2002) Identification of genes differentially regulated by glucocorticoids and progestins using a Cre/*loxP* mediated retroviral promoter-trapping strategy. Journal of Molecular Endocrinology 28(3):177-92

# Identification of genes differentially regulated by glucocorticoids and progestins using a Cre/*loxP*-mediated retroviral promoter-trapping strategy

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## Abstract

Glucocorticoids and progestins are two classes of steroid hormone with very distinct biological functions. However, the glucocorticoid receptor (GR) and the progesterone receptor (PR) share many structural and functional similarities. One way that glucocorticoids and progestins can exert different biological effects is through their different abilities to regulate the expression of certain target genes. A strategy employing a retroviral promoter-trap and Cre/*loxP*-mediated site-specific recombination has been developed to identify genes that are differentially regulated by glucocorticoids and progestins. A mouse fibroblast cell line (4F) stably expressing both GR and PR and containing a single copy of a multifunctional selection plasmid is generated. This line is transduced with a self-inactivating retroviral promoter-trap vector carrying coding sequences for Cre-recombinase (Cre) in the U3 region. Integration of the provirus places Cre expression under the control of a genomic flanking sequence. Activation of Cre expression from integration into active genes results in a permanent switch between the selectable marker genes that converts the cells from neomycin-resistant to hygromycin-resistant. Selection for hygromycin resistance after hormone treatment yields recombinants in which Cre sequences in the U3 region are expressed from hormone-inducible upstream cellular promoters. Because Cre-mediated recombination is a permanent event, the expression of the selectable marker genes is independent of ongoing Cre expression. Thus this system permits the identification of genes that are transiently or weakly induced by hormone.

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## Introduction

Glucocorticoids and progestins are two classes of steroid hormones with very distinct and complex biological functions.

Many biological actions of glucocorticoids in mammals have been described (Porterfield 1996). These include: (i) regulation of metabolism, (ii) anti-inflammatory and immunosuppressive actions, (iii) stimulation of bone loss by stimulating bone resorption and inhibiting bone formation, (iv) catabolic actions in muscle, skin and connective tissue, (v) actions in the kidney, cardiovascular and gastrointestinal system, and (vi) promotion of milk protein production in the mammary gland.

Abnormal function of glucocorticoids leads to complicated and pleiotropic symptomology; adrenocortical insufficiency causes Addison's disease whereas adrenocortical excess results in Cushing's syndrome. Thus, appropriate glucocorticoid action is critical to the maintenance of homeostasis. In addition, glucocorticoids have been widely employed as pharmaceuticals. For example, the immunosuppressive properties of glucocorticoids have been exploited to treat chronic inflammatory diseases such as asthma, rheumatoid arthritis, systemic lupus erythematosus, and transplant rejection. Glucocorticoids are also used in the treatment of leukemias and lymphomas. Nonetheless, in many cases, the molecular mechanisms

underlying the physiological and pharmacological actions of glucocorticoids are poorly understood.

Progestins play major physiological roles, particularly in female reproductive function (Graham & Clarke 1997). These include: (i) release of mature oocytes from the ovary, facilitation of implantation, maintenance of pregnancy by promotion of uterine growth and suppression of myometrial contractility, (ii) promotion of lobular-alveolar development in the mammary gland in preparation for milk secretion and suppression of milk protein synthesis before parturition, (iii) mediation of signals required for sexually responsive behavior, and (iv) prevention of bone loss. Antiprogestins have been applied to prevention of breast cancer and for safe abortion. However, as for glucocorticoids, the mechanisms that tie these biological actions of progestins in target tissues to genes regulated by progestins are not well defined.

Despite the different spectrum of physiological actions mediated by glucocorticoids and progestins, the respective receptors, glucocorticoid receptor (GR) and progesterone receptor (PR), are closely related members of the nuclear receptor family of transcription factors (Thornton 2001). GR and PR share many similar structural and functional characteristics. First, they have a similar domain structure. Both receptors consist of a nonhomologous (<15% sequence identity) N-terminal domain containing a ligand-independent transcription activation function (AF-1), a highly conserved (86% sequence identity) zinc finger DNA-binding domain (Hollenberg *et al.* 1985, Misrahi *et al.* 1987, Evans 1988, Kastner *et al.* 1990), a hinge region where the major nuclear-localization signal is located, and a homologous (54% sequence identity) ligand-binding domain containing a ligand-dependent transcription activation domain (AF-2). PR naturally exists as two isoforms, PR-B and PR-A, that are transcribed from two promoters in a single gene (Kastner *et al.* 1990, Sartorius *et al.* 1994). The only difference between PR-A and PR-B is that PR-A lacks 164 N-terminal residues that contain a unique activation function (AF-3). Secondly, the high sequence identity of the DNA-binding domains of the two receptors indicates that they have little or no ability to distinguish individual target sites (Lieberman *et al.* 1993). Both GR and PR bind as homodimers to an inverted repeat (optimal sequence PuGNACA) separated by 3 bp (Hynes *et al.* 1983, Payvar *et al.*

1983, Scheidereit *et al.* 1983, Cato *et al.* 1986, Lieberman *et al.* 1993). Thirdly, they share a similar mode of activation. In the absence of ligand, the receptors form an inactive complex with molecular chaperones. After ligand binding, the receptors dissociate from the chaperones and undergo dimerization (Pratt & Toft 1997). The dimerized receptors bind to target promoters, recruit coactivators and general transcription machinery, leading to regulation of transcription from target genes (Gronemeyer 1991, Tsai & O'Malley 1994). In addition, a number of natural and synthetic ligands bind to both receptors.

How can two receptors with such remarkable similarities mediate such dramatically different biological functions? One mechanism is the tissue-specific expression of receptors (Strahle *et al.* 1989). GR is broadly expressed in many tissues, but PR has a restricted pattern of expression that includes ovary, uterus, mammary gland, brain and bone (Graham & Clarke 1997). However, in certain tissues that express both GR and PR, these two hormones exert opposite effects under certain circumstances. For example, in mammary gland, glucocorticoids promote milk protein synthesis and lactation (Doppler *et al.* 1989, Groner *et al.* 1994, Groner & Gouilleux 1995), while progestins inhibit milk production and secretion (Graham & Clarke 1997). In bone, glucocorticoids stimulate bone resorption (Lane & Lukert 1998, Rackoff & Rosen 1998, Ziegler & Kasperk 1998) while progestins prevent bone loss (Nomura *et al.* 1989, Prior 1990).

We propose that glucocorticoids and progestins must exert different biological effects, at least in part, through their different abilities to regulate the expression of certain target genes. There could be multiple potential mechanisms for this differential gene regulation. (i) Receptor-specific binding to certain hormone response elements (HREs) in the target promoters (Nelson *et al.* 1999). In the context of multiple GR/PR-binding sites that together make up an HRE, mutation of a given site can differentially affect glucocorticoid and progesterone responses (Thackray *et al.* 1998). (ii) Receptor-specific abilities to remodel chromatin structure. For example, the different abilities of GR and PR to activate a stably integrated mouse mammary tumor virus (MMTV) promoter at certain chromosomal locations is due to the different abilities of GR and PR to remodel the chromatin structure

(Archer *et al.* 1994, Bartsch *et al.* 1996, Beato 1996, Truss *et al.* 1996, Smith *et al.* 1997, Lambert & Nordeen 1998). (iii) Receptor-specific coactivators and/or corepressors. Many activated nuclear receptors function by recruiting cofactors that bridge the steroid receptors and the general transcription machinery (Horwitz *et al.* 1996, Glass *et al.* 1997). There could be receptor-specific cofactors that mediate receptor-specific transcription regulation. (iv) Receptor-specific interactions with other transcription factors or receptor-specific crosstalk with other signaling pathways. There are increasing examples of ligand-dependent and -independent crosstalk of steroid receptors with other signaling pathways (Beato & Sanchez-Pacheco 1996). These interactions are probably sources of receptor-specific interactions that result in receptor-specific target gene regulation (Boonyaratanakornkit *et al.* 2001). The different abilities of GR and PR to regulate target genes may be mediated through the divergent part of the receptors, particularly the N-terminal domain.

Many of the studies on the mechanisms of GR and PR action have been performed using the MMTV promoter, but only a handful of cellular promoters that are regulated by GR or PR have been identified. In particular, there is a paucity of cellular promoters reported to be differentially regulated by GR and PR. In this study, we have utilized a promoter-trapping system to conduct a genome-wide search for the genes that can be induced by glucocorticoids or progestins, particularly those that can be differentially induced by the two hormones. The systematic identification of such genes and the characterization of the mechanisms that underlie differential hormone regulation will enhance our understanding of steroid physiology and give insight into the larger question of differential gene regulation by different members of transcription factor families.

## Materials and Methods

### Plasmid construction

Plasmid ppgklxtkneo/hygro and ppgklxtkneo/luc were constructed by modifying plasmid ppgklxtkneoIL3 (a generous gift from Dr Harald von Melchner) (Russ *et al.* 1996). In ppgklxtkneo/hygro, hygromycin phosphotransferase gene was sub-

cloned from plasmid pCMVhygro into ppgklxtkneoIL3. In ppgklxtkneo/luc, firefly luciferase gene was subcloned from plasmid pXP2 (Nordeen 1988) into ppgklxtkneoIL3. U3 Cre retroviral vector pGgU3 Creen(−) was a gift from Dr Harald von Melchner (Russ *et al.* 1996). Plasmid pCMVCre was constructed by cloning Cre gene from pGgU3 Creen(−) into pCMV6C vector (Chapman *et al.* 1991).

### Cell culture and transient transfection

The 4F and derived cell lines were maintained in Minimum Essential Medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT, USA), 10 mM Hepes, and nonessential amino acids. Transient transfections of 4F-derived cell lines were performed using a diethylaminoethyl/dextran method as previously described (Nordeen *et al.* 1998). After 20–24 h, cells were treated with vehicle, dexamethasone (100 nM) or R5020, a synthetic progestin, (10 nM). Cells were harvested 44–48 h after treatment. Cell extracts were prepared by first washing the cells, harvesting them in 0.5 ml cell lysis buffer, then pelleting debris (Nordeen *et al.* 1998). For luciferase assays, 25 µl soluble lysate were used and for β-galactosidase assays 2.5 µl. Luciferase and β-galactosidase assays were assessed using a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Sparks, MD, USA) as previously described (Nordeen *et al.* 1998). Data are reported as luciferase activity normalized to β-galactosidase activity in the same transfection.

The PA317 amphotropic packaging cell line was maintained in Dulbecco's Modified Eagle's Medium with high glucose (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone).

The T47D/A1–2 cell line was maintained in Minimum Essential Medium supplemented with 5% fetal bovine serum (Hyclone), 10 mM Hepes, nonessential amino acids and 200 µg/ml G418.

### Generation of 4 Ftkneo/hygro21 clonal cell line

Plasmid ppgklxtkneo/hygro was stably transfected into 4F cells by electroporation as described (Baum *et al.* 1994). Cells resistant to G418 (1 mg/ml) were cloned and subcloned. Genomic DNA Southern

blot analyses were performed to determine the copy number and integrity of the tkneo/hygro insert in each clone. To test integration copy number, a restriction enzyme that cuts only once in the reporter construct (BamHI) was used to digest the genomic DNA. To test insert integrity, a restriction enzyme that cuts the plasmid both upstream and downstream of the tkneo/hygro cassette (AlwNI) was used to digest the genomic DNA. Digested DNA was separated by agarose gel electrophoresis and transferred to Hybond N<sup>+</sup> nylon membrane (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The membrane was probed with either neomycin-resistance gene or hygromycin-resistance gene. One of the clones that contain a single copy of the full-length ppgklxtkneo/hygro cassette (4 Ftkneo/hygro21) was selected.

### U3 Cre virus packaging and infection

The U3 Cre retroviral vector was stably transfected into an amphotropic packaging cell line PA317 (Miller & Buttimore 1986) by a calcium phosphate coprecipitation method. Because the U3 Cre plasmid lacks a selectable marker, plasmid pRSV-neo was cotransfected at 1/10 amount of the retroviral plasmid. Stable clones were selected in G418 media (1 mg/ml). Clones were screened for the existence and integrity of Cre gene by PCR amplification of genomic DNA followed by Southern blot analysis. The ability of these packaging cell clones to produce U3 Cre retrovirus was tested by infecting the 4 Ftkneo/hygro21 cells and selecting for hygromycin resistance. Viral titer was determined in the same way. Because the U3 Cre provirus lacks a constitutively expressed drug-resistance marker, viral titers were derived by multiplying the number of hygromycin-resistant colonies by the average frequency of integrations that enable U3 gene activation of other similar gene-trap vectors (von Melchner & Ruley 1989, von Melchner *et al.* 1990). The packaging cell clone (clone 4) that produced U3 Cre retrovirus at the highest titer ( $>10^7$  cfu/ml) was selected and used to prepare viral supernatant. Viral infection of the 4 Ftkneo/hygro21 cells was performed at a multiplicity of infection (MOI) of 0.5 to ensure single viral integration in each cell. A library consisting of approximately  $4 \times 10^6$  independent proviral integrations was generated.

### Sequential drug selection procedure

Sequential drug selections were performed to isolate clonal cell lines representing trapped, hormone-inducible and differentially hormone-inducible genes as described in the later Fig. 3. Retrovirus-transduced cells were first placed under G418 (1 mg/ml) selection for 5 days. Those cells in which the Cre gene is integrated downstream of a constitutively active cellular will be eliminated due to the expression of Cre and thus the deletion of the tkneo gene. The survivors were split into two groups in media without G418. One group was treated with R5020 (10 nM) and the other with dexamethasone (100 nM) for 24 h. The cells are then subjected to hygromycin (500 µg/ml) selection to select for cells in which Cre expression has been induced by hormone induction. Thus this selection will enrich for cells in which the Cre gene was integrated downstream of a progestin-inducible promoter (group A) or a glucocorticoid-inducible promoter (group B). To maximize the identification of genes preferentially induced by one of the two hormones, an additional step was added to the selection scheme. After the initial G418 selection to eliminate constitutively expressed promoters, another selection was added to eliminate promoters induced by progestins (group C) or glucocorticoids (group D). This was done by adding the appropriate hormone for 24 h and continuing G418 selection for 5 days. Survivors in the progestin-treated group C were removed from G418 and treated with dexamethasone for 24 h. Similarly, the glucocorticoid-treated group D was removed from G418 and treated with R5020 for 24 h. The two groups were then placed under hygromycin selection to enrich for cells expressing Cre under the control of the appropriate hormone. A total of 120 clones, 40 each from groups A and B, and 20 each from groups C and D, were isolated for further analysis.

### Amplification and cloning of proviral upstream sequences (inverse PCR)

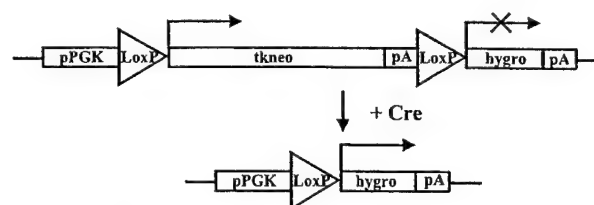
Genomic DNAs from U3 Cre-infected 4 Ftkneo/hygro21 cell clones were digested with restriction enzyme (MseI or HinfI) and ligated at 5 µg/ml to obtain circular molecules. After cleavage with PvuII, 1 µg of each sample was used for PCR. PCR reactions were performed using an Expand high

fidelity PCR system (Roche Diagnostics Corporation, Indianapolis, IN, USA) as previously described (Russ *et al.* 1996). Amplification products were cloned into the TA cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced.

### Semi-quantitative RT-PCR

Total RNA was isolated from each sample using a Purescript RNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. RNA was treated with RNase-free DNaseI (Ambion, Inc., Austin, TX, USA) to remove genomic DNA contamination. RNA was reverse transcribed into single-stranded cDNA using random primers and M-MLV reverse transcriptase (Life Technologies). For semi-quantitative PCR, first, the best cycle number for linear amplification of the cDNA using each gene-specific primer pairs was determined. One cDNA sample was serially diluted and used as template for PCR, which was performed with Taq DNA polymerase (Promega, Madison, WI, USA). PCR products were run on agarose gel and stained with 'SYBR Gold' Nucleic Acid fluorescent stain (Molecular Probes, Inc., Eugene, OR, USA). Each PCR product was quantitated with the Molecular Dynamic STORM 860 system. The PCR output was plotted against the relative cDNA input and the cycle that gave the best linear relationship was chosen to PCR amplify each experiment sample. The linear standard cDNA and the cDNA from each experiment sample were PCR amplified side by side. The PCR output for each sample was quantitated and used to calculate the input cDNA amount using the linear relationship derived from the standard.

Primers used for amplifying  $\beta$ -actin were from Promega. The cre gene-specific primer used to amplify cellular-cre fusion transcript was 5'-GGTGTACGGTCAGTA-3'. Primers used for amplifying genes identified in the indicated clonal cell line are as follows. Clone 32 gene: 32-A 5'-GAAGATCTGAACTCCAAGGT-3', 32-B 5'-ATACCCACAAAGGAGATCTG-3'; clone 43 gene: 43-A 5'-ACTAGCCAGTATAGTGCT-3', 43-B 5'-CATGTATATCTCAAAAAGTG-3'; clone 42 gene: 42-A 5'-CTTCTAAAGCGGTCCAGAGC-3', 42-B 5'-CATGACCAAGAAAGTGT CAC-3'; clone 66 gene: 66-A 5'-CCTGTGTTC



**Figure 1** Schematic of the multifunctional selection plasmid (ppgklxtkneo/hygro) before and after Cre-mediated recombination at *loxP* sites. Functional components are labeled as follows: pPGK, mouse phosphoglycerate kinase promoter; tkneo, herpes simplex virus 2 thymidine kinase (*tk*)-neomycin phosphotransferase (*neo*) fusion gene; hygro, hygromycin-B phosphotransferase; pA, a pair of polyadenylation signals from the growth hormone gene; *loxP*, Cre recombination target sequences.

TCCTGGACCT-3', 66-B 5'-GTCCTGAGAACT GGAATCTG-3'

## Results

### Establishment of a Cre/*loxP*-mediated retroviral promoter-trapping system

In order to study the actions of both GR and PR in the same cells, a mouse fibroblast-derived cell line, 4F, was generated by stably introducing exogenous PR-B expression into the glucocorticoid-responsive Ltk<sup>-</sup> fibroblast cells as described (Thackray *et al.* 1998). Although 4F cells are responsive to both glucocorticoids and progestins, PRs are at least 10-fold more abundant than GRs in each 4F cell (Thackray *et al.* 1998).

To obtain a cell line that can sensitively detect Cre-recombinase expression, the multifunctional reporter plasmid (ppgklxtkneo/hygro) (Fig. 1) was stably transfected into 4F fibroblasts by electroporation. The ppgklxtkneo/hygro reporter plasmid consists of two, tandemly arrayed selective marker genes that are transcribed from a *pgk* promoter. The 5' selective marker gene encodes for a fusion protein between herpes simplex virus 2 thymidine kinase (*tk*) and the neomycin phosphotransferase (*neo*), and is flanked by two direct-repeats of the *loxP* recombination target site (Russ *et al.* 1996). The 3' selective marker gene encodes for hygromycin phosphotransferase (*hygro*) and is located downstream of the second *loxP* site. In the absence of Cre, the *tkneo* gene is expressed from the constitutive *pgk* promoter. The *hygro* gene does not



have a promoter immediately upstream and chimeric transcripts initiated at the *pgk* promoter are suppressed by two tandem copies of the bovine growth hormone polyadenylation sequence upstream of *hygro*. Therefore, the cells containing a single copy of ppgklxtkneo/*hygro* are G418-resistant but hygromycin-sensitive. However, when Cre is expressed, it will catalyze recombination between the two *loxP* sites resulting in the deletion of *tkneo*. This places *hygro* immediately downstream of the *pgk* promoter rendering the cells G418-sensitive but hygromycin-resistant (Fig. 1). Thus, 4F cells with a stably integrated ppgklxtkneo/*hygro* selection plasmid report even transient expression of Cre by undergoing a permanent switch in their drug-resistance phenotype.

The 4F cells were stably transfected with the selection plasmid. A G418-resistant clone containing a single copy plasmid (4 Fneo/*hygro*21) was selected for further analysis (see Materials and Methods). First, we determined whether the 4 Fneo/*hygro*21 cells were sensitive to hygromycin by placing  $5 \times 10^5$  cells under hygromycin (500  $\mu\text{g}/\text{ml}$ ) selection. Because no colony formed within 12 days, we concluded that neither leaky *hygro* translation nor spontaneous recombination occurred in these cells at levels that would interfere. Secondly, we determined whether the 4 Fneo/*hygro*21 cells would become hygromycin-resistant and neomycin-sensitive after Cre expression. An expression plasmid, pCMVCre, was transiently transfected into the cells by a calcium phosphate coprecipitation method. Cells were selected for hygromycin (500  $\mu\text{g}/\text{ml}$ ) resistance. Then the hygromycin-resistant cells were pooled and placed under G418 (1 mg/ml) selection for 12 days to test neomycin sensitivity. Because all the hygromycin-resistant cells were neomycin-sensitive, we concluded that the cells could undergo a clean drug-resistance phenotype switch after Cre expression.

In this gene-trapping system, we employed a self-inactivating retroviral gene-trap vector (U3 Cre) in which a promoterless Cre gene is inserted in the U3 region of an enhancer-deleted long terminal repeat (LTR) (Fig. 2). Viral replication and LTR-mediated duplication place the promoter-less Cre sequence just 30 nucleotides downstream of the flanking cellular DNA (Fig. 2) (von Melchner & Ruley 1989, von Melchner *et al.* 1990, Russ *et al.* 1996). Therefore, Cre is not expressed unless the virus integrates into an active cellular promoter. In

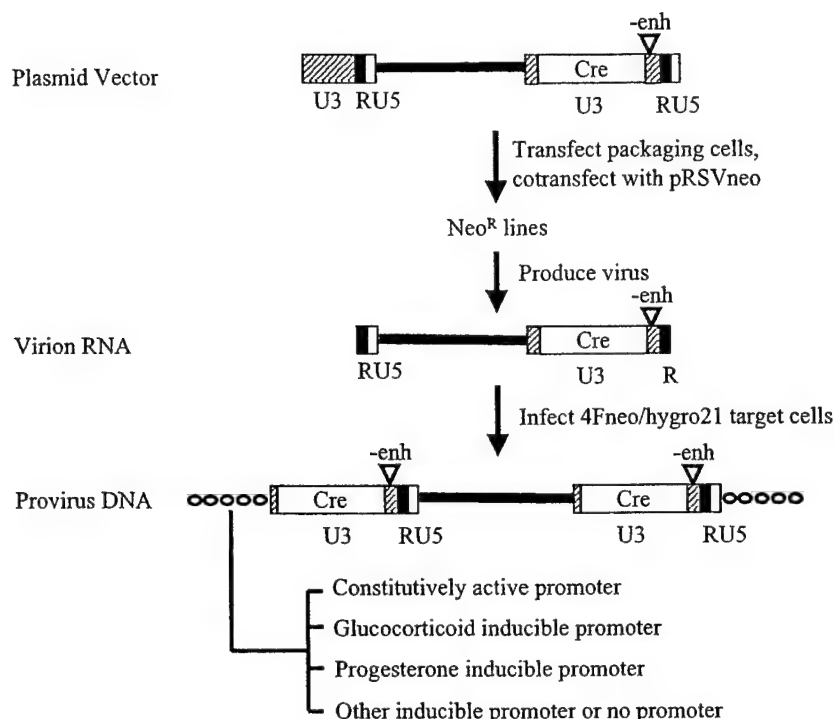
this work, we employ this feature to identify hormone-regulated promoters.

#### Isolation of cell lines representing trapped, hormone-inducible and differentially hormone-inducible promoters

The U3 Cre plasmid was stably transfected into an amphotropic packaging cell line PA317 (Miller & Buttimore 1986). Supernatants from lines producing high titers of recombinant virus were used to infect 4 Fneo/*hygro*21 cells (see Materials and Methods) (von Melchner & Ruley 1989, von Melchner *et al.* 1990) resulting in a library consisting of approximately  $4 \times 10^6$  independent proviral integration events. Clonal cell lines were isolated following sequential drug selections as outlined in Fig. 3. As detailed in Materials and Methods, the selection scheme for cell clones of group A enriches for promoters induced by progestins whereas group B is enriched for promoters induced by glucocorticoids. Trapped promoters preferentially induced by glucocorticoids vs progestins are enriched in cell clones of group C and conversely promoters preferentially induced by progestins are trapped in the scheme giving group D cell clones. Forty cell lines from each of group A and group B, and an additional 20 cell lines from each of group C and group D were isolated for further analysis.

In the clonal cell lines with a stably integrated U3 Cre provirus, typically there are two cre transcripts: a cellular-proviral fusion transcript initiating from the upstream cellular promoter and terminating at the polyadenylation site of the 5' LTR, and a 'viral genomic' transcript initiating from the 5' proviral LTR and terminating at the polyadenylation site of the 3' LTR (Russ *et al.* 1996). The double cre transcripts can complicate the analysis of the selected cell lines since the viral genomic transcript can obscure the cre transcript from the cellular promoter. However, translation of Cre from the 'viral genomic' transcript within the 3' LTR is unlikely to be due to multiple short open reading frames positioned between the LTRs. Furthermore, several previous studies have shown that, in most cases, activation of the promoter-trap is associated with the translation of Cre from the cellular-proviral fusion transcripts (von Melchner & Ruley 1989, von Melchner *et al.* 1990, Reddy *et al.* 1991, Chang *et al.* 1993, Russ *et al.* 1996).





**Figure 2** Structure and replication of the U3 Cre retroviral vector. Functional components are labeled as follows: Cre, modified bacterial phage P1 Cre-recombinase; U3, R and U5 are segments of the viral LTR. '-enh' indicates 'enhancer deleted'. Infection of 4 Fneo/hygro21 target cells generates provirus in which the 5' cre-coding sequence is immediately downstream of cellular sequence. Neo<sup>R</sup>, G418-resistant.

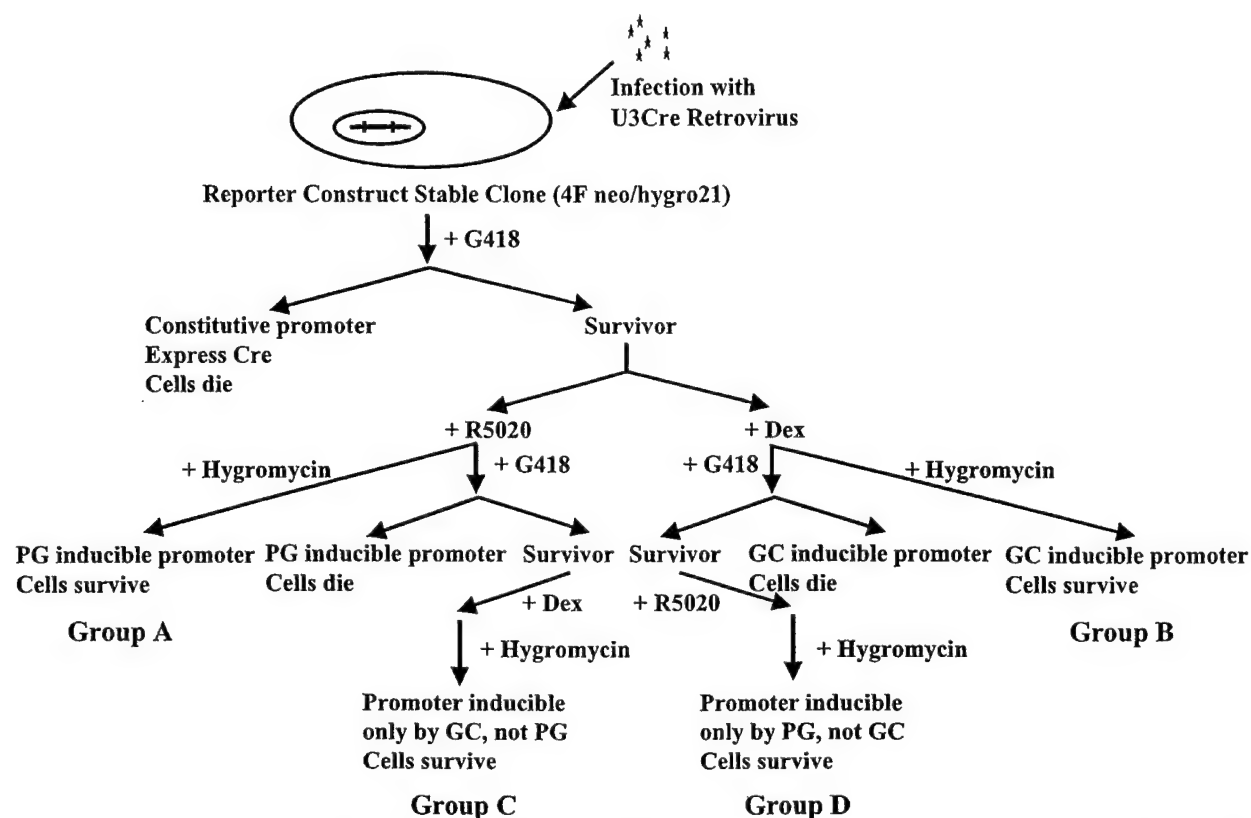
To determine the expression pattern of the gene represented in each clonal cell line isolated, we developed a reporter assay to quantitate the Cre protein level based on Cre-recombinase activity (Fig. 4). A reporter plasmid (ppgklxtkneo/luc) (Fig. 4A) was constructed in which the *hygro* gene of the multifunctional selection plasmid was replaced by luciferase (*luc*). Luciferase expression from this plasmid was dependent on Cre expression. A titration of Cre expression vector demonstrated that the higher the input of Cre, the higher the output of luciferase expression (Fig. 4B). Therefore, this sensitive and quantitative luciferase reporter strategy can determine the exact regulation pattern of the trapped promoters in each isolated cell clones and allow the identification of the lines that represent genes that are differentially regulated by the two hormones.

Each clonal cell line with potentially hormone-regulated, trapped promoters was transfected with ppgklxtkneo/luc and treated with dexamethasone or R5020 24 h later. The induction of Cre activity

was assessed by the deletion of sequences flanked by *loxP* sites as measured by the appearance of luciferase activity. In 58 cell lines, luciferase expression was induced by either glucocorticoids or progestins by more than 2-fold. In 19 cell lines, luciferase expression was differentially regulated by the two hormones by more than 1.5-fold (Fig. 4C).

### Hormone regulation of selected promoters

In order to determine whether the endogenous cellular genes trapped in the clonal cell lines are indeed hormone-regulated, genomic DNA sequences upstream of the proviral integration site of several clonal isolates were retrieved by inverse PCR and sequenced, ranging from 154 to 874 bp (Fig. 5, also see Materials and Methods) (von Melchner *et al.* 1990, Russ *et al.* 1996). Sequences from all four clones tested showed a typical cellular DNA-provirus junction (Fig. 5B) (Varmus 1988). Blast searches using different databases revealed



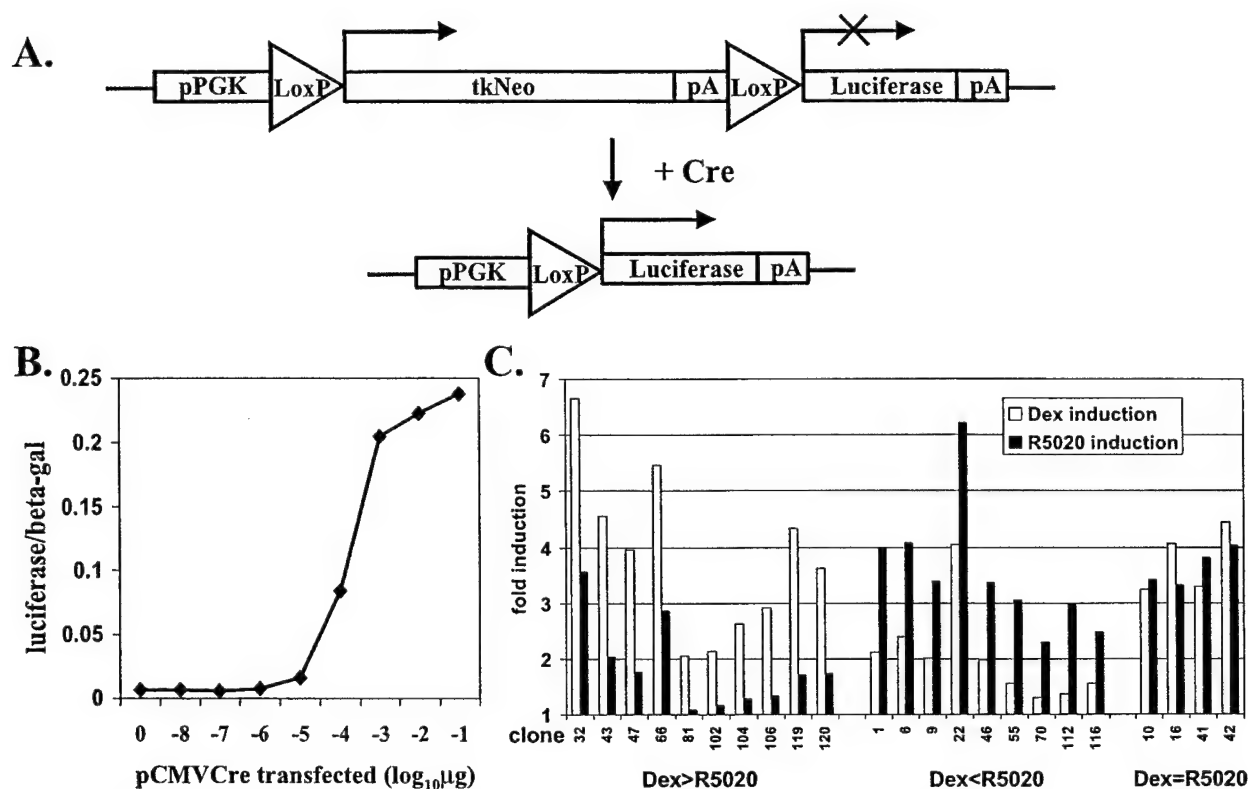
**Figure 3** Schematic illustration of the sequential selection procedure to identify 4F cell lines representing a trapped promoter that is induced or differentially induced by glucocorticoids and progestins. GC, glucocorticoids; PG, progestins; Dex, dexamethasone, a synthetic glucocorticoid; R5020, a synthetic progestin. Specifics on the selections are detailed in the Material and Methods.

that the entire sequence upstream of the proviral integration site in clone 43 (441 bp) is 98% identical with the expressed sequence tag (EST) ic84f11.x1 in the cDNA library 'Melton Normalized Mixed Mouse Pancreas 1 N1-MMS1' (dbEST ID 9280663, GenBank accession number B1438077). However, the sequences upstream of the proviral integration sites in clone 32, 42 and 66 showed no significant homology to known genes or ESTs at the time of analysis (see Discussion). These sequences have been deposited in GenBank and accession numbers are AF465703 (for clone 32 gene), AF465704 (for clone 42 gene) and AF465705 (for clone 66 gene).

Three of the four proximal flanking sequences (clone 32, 42 and 43) were able to be amplified by RT-PCR from the RNA isolated from the parental cell line (4F), suggesting that the upstream sequences are within an exon of the gene (Fig. 6 and 7). The primers designed for the upstream

sequences of clone 66 did not give a RT-PCR product even though they can amplify the genomic DNA (data not shown). The integration site in this clone may not be in an exonic sequence or the 5' primer could be upstream of the transcription start site.

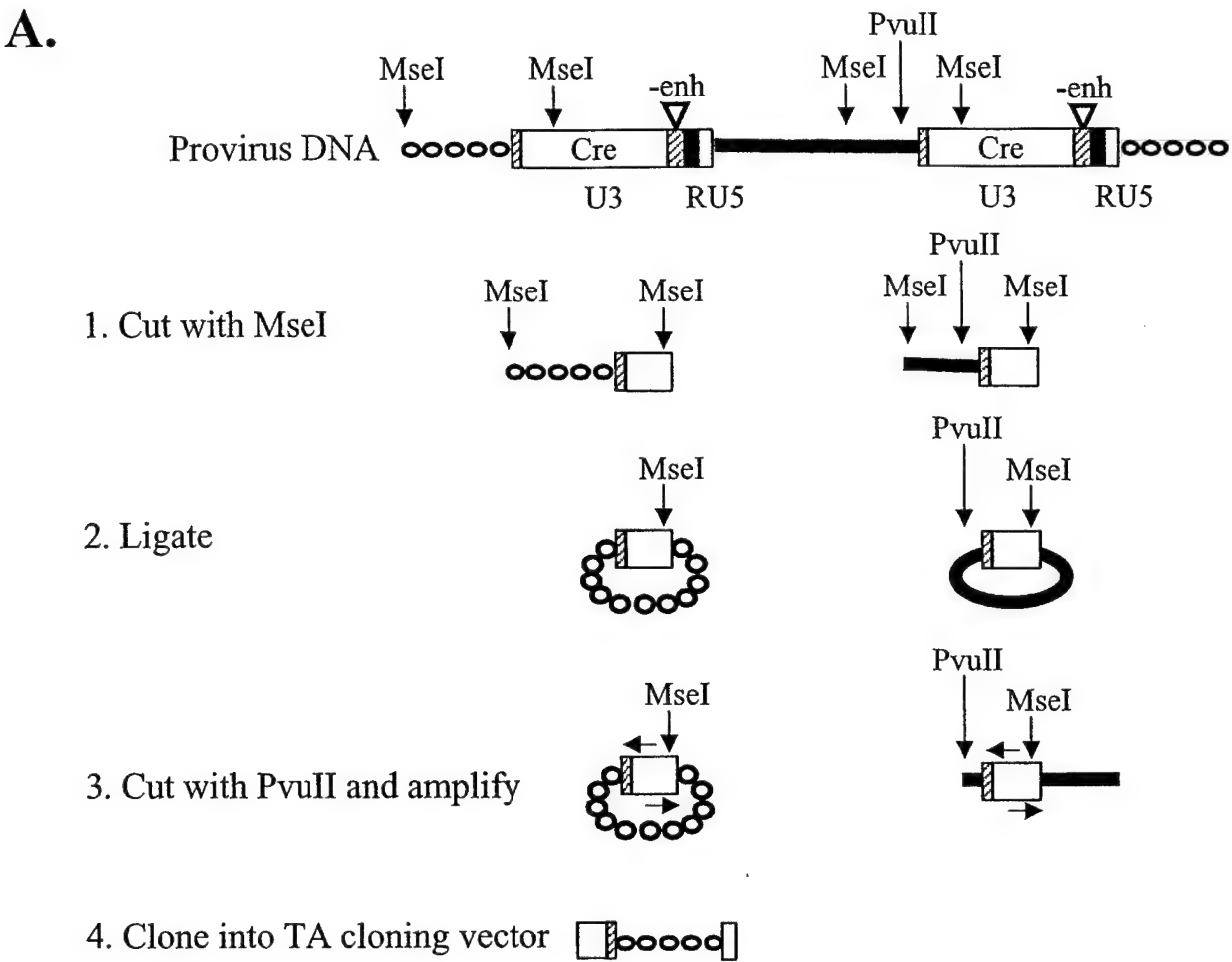
The mRNA level of the gene represented by clone 32 (clone 32 gene) in the parental cell line (4F) was measured by semi-quantitative RT-PCR (see Materials and Methods). Serially diluted cDNAs were amplified by PCR to select the cycle number that gave the best linear relationship between the cDNA input ( $x$  value) and the PCR output ( $y$  value) for each specific primer set (Fig. 6A and 6B). Then the cDNAs in each hormone-treated sample were amplified at this cycle number, with the linear standard run at the same time. The PCR output in each sample was quantitated and the amount of cDNA input was calculated using the linear relationship derived from the standard



**Figure 4** Quantitation of Cre protein expression in clonal cell lines by transfection of ppgklxtkneo/luc. (A) Schematic of ppgklxtkneo/luc reporter construct before and after Cre-mediated recombination at *loxP* sites. Gene sequences are labeled as following: pPGK, mouse phosphoglycerate kinase promoter; tkneo, herpes simplex virus 2 thymidine kinase (*tk*)–neomycin phosphotransferase (*neo*) fusion gene; pA, a pair of polyadenylation signals from the growth hormone gene; *loxP*, Cre recombination target sequences. (B) A dose curve demonstrating that relative levels of Cre activity can be analyzed by recombination of ppgklxtkneo/luc and concomitant production of luciferase activity. Increasing amounts of the expression plasmid pCMVCre was transiently transfected into the 4 fneo/hygro21 cell line. A constant amount of the reporter tkneo/luc (1 μg/ml) and an internal control plasmid pCMVβ-gal (0.1 μg/ml) were cotransfected. Cells were harvested 72 h later. The result is shown as luciferase activity normalized to β-galactosidase activity. (C) Identification of clonal cell lines that represent promoters differentially regulated by glucocorticoids and progestins by quantitating Cre protein expression. The reporter plasmid ppgklxtkneo/luc (1 μg/ml) was transiently transfected into each clonal cell line with an internal control plasmid pCMVβ-gal (0.1 μg/ml). After 20–24 h, cells were treated with vehicle, dexamethasone (Dex, 100 nM) or R5020 (10 nM). Cells were harvested 48 h after treatment. The results are illustrated as the fold of induction of the luciferase activity normalized to β-galactosidase activity. Shown are 19 cell lines that represent promoters differentially regulated by the two hormones by more than 1.5-fold: Dex>R5020 indicates that the promoters are preferentially induced by dexamethasone, Dex<R5020 indicates that the promoters are preferentially induced by R5020. In addition, four examples of the cell lines that represent promoters induced by both hormones by more than 2-fold are also shown (Dex=R5020).

(Fig. 6C). The result demonstrated that clone 32 gene was preferentially induced by glucocorticoids (5-fold) rather than by progestins (2.5-fold). Although this is only a 2-fold difference in the induction, it is more impressive when one considers that PRs are 10-fold more abundant than GRs in these cells. Expressions of the genes represented by the other two clones were quantitated in the same way (Fig. 7). Clone 43 gene was also preferentially

induced by glucocorticoids. Glucocorticoids induced gene 43 by 2-fold while progestins gave no induction or a slight inhibition. Clone 42 gene was induced by both hormones to a similar level. For all three clones, the mRNA regulation patterns of the endogenous genes agree with the Cre protein regulation patterns shown in Fig. 4C, suggesting that the genes identified using this strategy are indeed regulated by hormones.



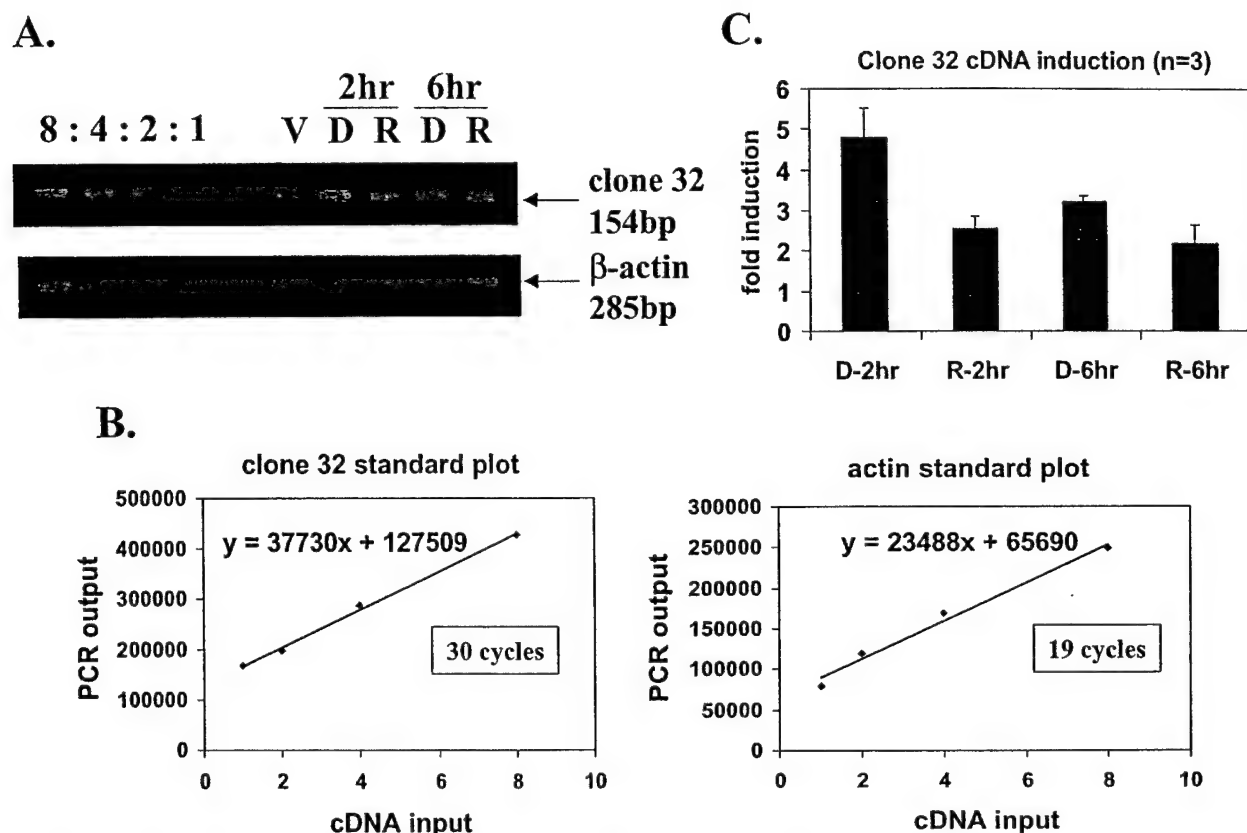
**B.**

Clone	Enzyme	Flanking DNA, nt	Cellular-Proviral Junction
32	MseI	154	CTCCTTTGTGGGTATTGAAAGACCCC
42	MseI	234	CTTTCTTGGTCATGATGAAAGACCCC
43	MseI	441	ATACATACATACATATGAAAGACCCC
66	Hinfi	874	TAATATTTACAGGGTGAAAGACCCC

**Figure 5** Retrieval of sequences upstream of the proviral integration site by inverse PCR. (A) Schematic of inverse PCR procedure. The restriction enzyme used in this example was MseI. Experimental details are given in Materials and Methods. (B) Summary of the upstream sequences retrieved from selected clonal cell lines. Proviral sequences are underlined.

For two clones (32 and 43), we further examined the expression of the cellular-cre fusion transcript to ascertain whether it is regulated by hormones in

the same fashion as the endogenous gene. Clonal cell lines 32 and 43 were treated with hormone for 2 h and total RNA was isolated. Semi-quantitative



**Figure 6** Quantitation of the mRNA level of clone 32 gene in 4F cells by semi-quantitative RT-PCR. (A) Parental cells (4F) were treated with vehicle (V), dexamethasone (D, 100 nM) or R5020 (R, 10 nM) for 2 or 6 h. Total RNA was isolated and used for semi-quantitative RT-PCR. The left four lanes show the standard (see Materials and Methods). (B) The linear relationship between the cDNA input and the PCR output for clone 32 gene (left) and the β-actin control (right) at the indicated PCR cycle. (C) Quantitation of clone 32 gene-specific cDNA normalized by β-actin-specific cDNA in each hormone-treated sample. The result is shown as fold of induction compared with vehicle-treated sample. The Figure depicts the results of three independent experiments. Error bars represent ±S.E.

RT-PCR analyses were performed with a 5' primer from the endogenous gene and a 3' primer from the cre sequence. A PCR product of the predicted size was amplified from cDNA. Furthermore, both fusion transcripts were preferentially induced by glucocorticoids compared with progestins, as was observed for the endogenous genes (Fig. 8).

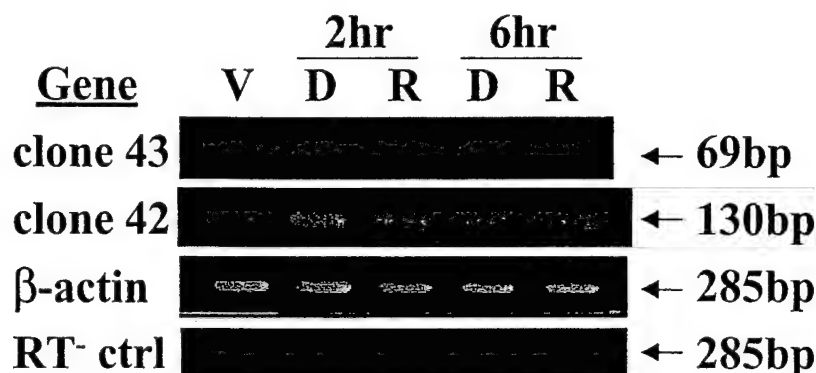
#### Glucocorticoid induction of clone 32 gene is dependent on GR and does not require *de novo* protein synthesis

Further characterizations of the glucocorticoid induction were performed using the clone 32 gene as an example. In order to assess the specificity of the induction of clone 32 gene by glucocorticoid, a dose-response study was performed. 4F cells were

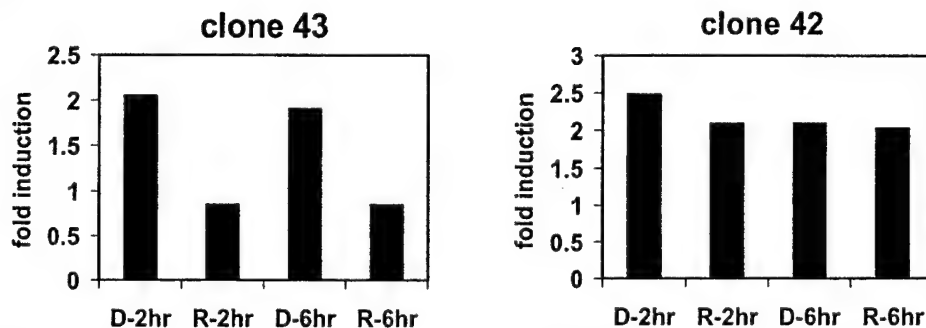
treated with increasing concentration of dexamethasone for 2 h. Total RNA was isolated from each sample and the expression of clone 32 gene was quantitated by semi-quantitative RT-PCR (Fig. 9A). The result demonstrated that even at a concentration as low as 1 nM, dexamethasone can induce the expression of the clone 32 gene, suggesting that the induction of clone 32 gene by glucocorticoid is specific.

In order to determine whether the induction of clone 32 gene by glucocorticoid is dependent on GR, 4F cells were treated with 100 nM RU486 or ZK112 in addition to hormone treatment for 2 h. RU486 is an antagonist that can block both GR and PR function, while ZK112 is a much stronger antagonist for PR than for GR (data not shown, D Henderson, unpublished observation).

**A.**



**B.**



**Figure 7** Quantitation of the mRNA level of genes represented by clone 43 and 42 in 4F cells by semi-quantitative RT-PCR. RT-PCR analyses were performed as described in Fig. 6 and in Materials and Methods. (A) Parental cells (4F) were treated with vehicle (V), dexamethasone (D, 100 nM) or R5020 (R, 10 nM) for 2 or 6 h. Total RNA was isolated and used for semi-quantitative RT-PCR. (B) Quantitation of each gene-specific cDNA normalized by β-actin-specific cDNA in each hormone-treated sample. The result is shown as fold of induction compared with vehicle-treated sample.

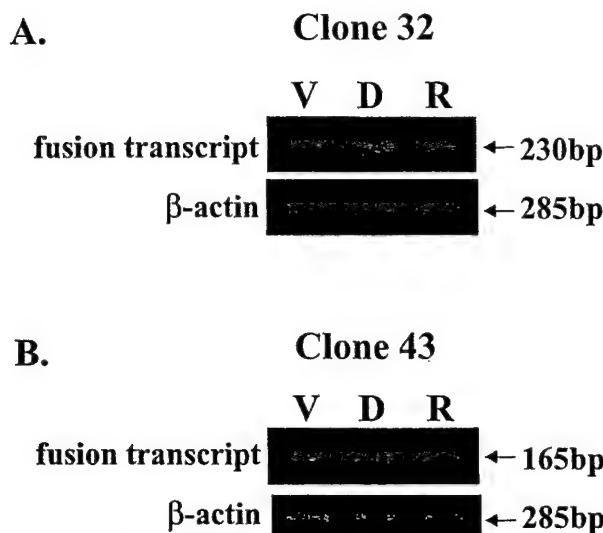
Quantitation of clone 32 gene expression by semi-quantitative RT-PCR (Fig. 9B) demonstrated that the induction of clone 32 gene by either dexamethasone or R5020 was completely abolished following RU486 treatment. In contrast, following ZK112 treatment, the induction by R5020 is abolished while induction by dexamethasone is only slightly reduced. These results indicate that the induction of clone 32 gene by glucocorticoid is dependent on GR.

In order to determine if the induction of clone 32 gene by glucocorticoid is a direct response, 4F cells were treated with *de novo* protein synthesis inhibitor cycloheximide for 15 min prior to

hormone treatment. Quantitation of clone 32 gene expression by semi-quantitative RT-PCR (Fig. 9C) demonstrated that the induction of clone 32 gene by dexamethasone was maintained following cycloheximide treatment. This suggests that the glucocorticoid induction is a direct response that does not require *de novo* protein synthesis.

#### Glucocorticoid induction of clone 32 gene is an early response and is cell-type specific

The kinetics of the hormone regulation of clone 32 gene in 4F cells was assessed by semi-quantitative RT-PCR (Fig. 10A). Glucocorticoid induction of



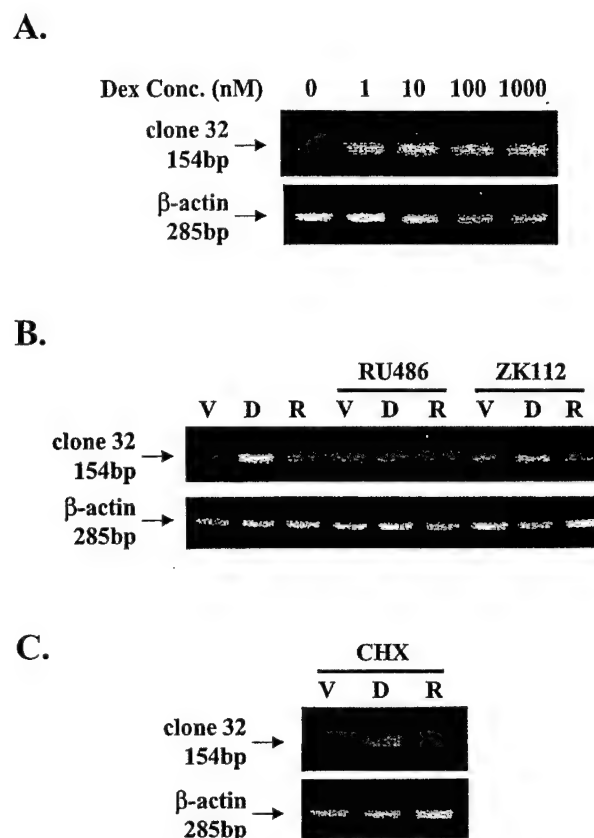
**Figure 8** Quantitation of cellular-cre fusion transcripts in clonal cell line 32 and 43 by semi-quantitative RT-PCR. Clonal cell lines 32 (A) and 43 (B) were treated with vehicle (V), dexamethasone (D, 100 nM) or R5020 (R, 10 nM) for 2 h. Total RNA was isolated and used for semi-quantitative RT-PCR.

clone 32 gene peaked as early as 30 min, again indicating that this is a direct response of hormone regulation. The hormone induction lasted for at least 22 h.

Hormone regulation of clone 32 gene in two other cell types was also assessed. L929 cells are mouse fibroblasts like the 4F cells and their parent line, Ltk<sup>-</sup>. L929 cells express only GR but not PR. Semi-quantitative RT-PCR assays demonstrated that the clone 32 gene was also induced by glucocorticoids in L929 cells (Fig. 10B). T47D/A1-2 cells were derived from T47D human mammary carcinoma cells and have been engineered to express high levels of rat GR (Nordeen *et al.* 1989, Moyer *et al.* 1993). We have previously estimated that these cells express about 100 000 molecules of GR per cell, about half the number of endogenously expressed PR. The clone 32 gene was induced by neither glucocorticoids nor progestins in T47D/A1-2 cells (Fig. 10C). These results indicate that the hormone regulation of clone 32 gene is cell-type specific.

## Discussion

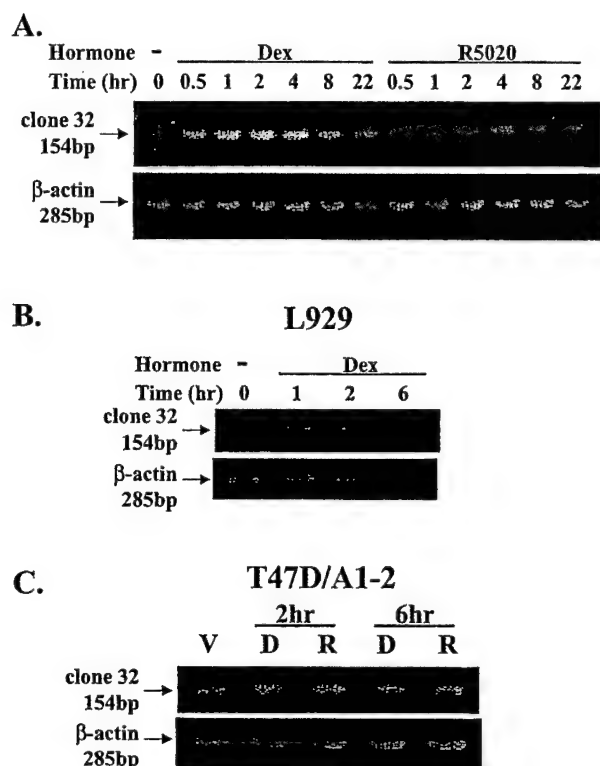
In this study, we employed a strategy that combines a retrovirally delivered promoter-trap and Cre/



**Figure 9** Glucocorticoid induction of clone 32 gene is dependent on GR and does not require *de novo* protein synthesis. (A) A dose-response assessment of the induction of clone 32 gene by glucocorticoid. 4F cells were treated with dexamethasone at indicated concentration for 2 h. Total RNA was isolated and used for semi-quantitative RT-PCR. (B) Glucocorticoid induction of clone 32 gene is dependent on GR. 4F cells were treated with 100 nM RU486 or ZK112 in addition to treatment with vehicle (V), dexamethasone (D, 100 nM) or R5020 (R, 10 nM) for 2 h. Total RNA was isolated and used for semi-quantitative RT-PCR. (C) Glucocorticoid induction of clone 32 gene does not require *de novo* protein synthesis. 4F cells were pretreated with cycloheximide (CHX, 40 µg/ml) for 15 min and then treated with vehicle (V), dexamethasone (D, 100 nM) or R5020 (R, 10 nM) for 2 h in the presence of cycloheximide. Total RNA was isolated and used for semi-quantitative RT-PCR.

*loxP*-mediated site-specific recombination to identify hormone-regulated genes, especially those that are differentially regulated by glucocorticoids and progestins. This strategy has several significant advantages. (i) It can detect transiently hormone-activated promoters. Since the Cre-mediated recombination at *loxP* sites is an irreversible event,





**Figure 10** Glucocorticoid induction of clone 32 gene is an early response and is cell-type specific. (A) The kinetics of the hormone regulation of clone 32 gene. 4F cells were treated with dexamethasone (Dex, 100 nM) or R5020 (10 nM) for the indicated time. Total RNA was isolated and used for semi-quantitative RT-PCR. (B, C) Hormone regulation of clone 32 genes in different cell lines. L929 cells (B) and T47D/A1-2 cells (C) were treated with vehicle (V), dexamethasone (D or Dex, 100 nM) or R5020 (R, 10 nM) for indicated times. Total RNA was isolated and used for semi-quantitative RT-PCR.

even transiently activated promoters can produce enough Cre-recombinase to create a permanent switch of drug-resistance phenotype. Thus screening can be accomplished even when a brief induction period is used to avoid cytotoxicity or induction of apoptosis that might result from a longer treatment (Russ *et al.* 1996). (ii) It is not strongly biased toward highly expressed genes. Little Cre expression is required to catalyze recombination at the *loxP* sites. Therefore, unlike many conventional strategies, e.g. cDNA library screening, RNA differential display, and microarray analysis, this Cre/*lox* selection strategy can detect weakly expressed genes. (iii) It allows discovery of unknown genes. (iv) It allows a

near-saturation screening of the whole genome. If we infect the target cell line with the U3 Cre retrovirus at an MOI of 0.5 to ensure single integration events and perform selections on  $10^7$  integrants, this will yield 1 integration per 300 bp, assuming random integration.

In addition to documenting this promoter-trapping system, we describe a sensitive and quantitative luciferase reporter strategy that can determine the regulation pattern of the trapped promoters in the isolated cell clones. Sequences upstream of the proviral integration site in selected clones were retrieved by inverse PCR. Hormone regulation of the endogenous genes identified in selected clones has been confirmed by semi-quantitative RT-PCR, indicating that many of the genes identified with these strategies are indeed hormone-regulated. Most significantly, we have successfully identified genes that are differentially regulated by two closely related transcription factors, the GR and the PR.

For three of the four genes analyzed, database searches of the sequences at the site of proviral integration reveal no homology to known genes or ESTs. This was not a great surprise, as the genes identified had extremely low basal expression. Therefore, identification of these transcription units might be easily missed by conventional gene discovery approaches. Another reason that cDNAs for these transcription units may not be found in the databases stems from the design of the U3 Cre retroviral construct that selects for integration sites in 5' nontranslated regions. An amber stop codon (TAG) is located 24 nucleotides upstream and in frame with the initiation codon AUG for Cre. This imposes a strong selection for integration events in which Cre provided the first initiating AUG in the resulting hybrid transcript (von Melchner *et al.* 1990). For mammalian genes, the average distance between transcription start site and the first AUG initiation codon is 50–100 nucleotides (Kozak 1987). The 5'-most part of the transcription unit is most likely to be absent from all but full-length cDNA clones in the databases.

In this study, a group of 19 clonal cell lines representing genes differentially regulated by glucocorticoids and progestins have been isolated. Two genes (clone 32 and 43 genes) have been confirmed to be preferentially induced by glucocorticoids compared with progestins using RT-PCR. Despite the disparate biological activities of the two



receptors, there is little information on what genes may be differentially regulated by the two receptors and how this may be accomplished. Our studies document a novel approach toward addressing this void. Further studies on the mechanisms underlying the differential regulation will enhance our understanding of how glucocorticoids and progestins function as specific physiological regulators or therapeutic agents.

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We would like to thank Dr Harald von Melchner for providing plasmids ppgklxtkneoIL3 and pGgU3 Creen(-), and Dr James DeGregori for insightful discussion. We also acknowledge the assistance of the DNA sequencing core of the University of Colorado Cancer Center. This work has been supported by grant award DK37061 from the National Institutes of Health to S K N and predoctoral fellowship award DAMD17-99-1-9445 from the Department of Defense to Y W.

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**Appendix B:** A copy of the published article

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## Overlapping but Distinct Gene Regulation Profiles by Glucocorticoids and Progestins in Human Breast Cancer Cells

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Glucocorticoids and progestins bind to receptors that share many structural and functional similarities, including virtually identical DNA recognition specificity. Nonetheless, the two hormones mediate very distinct biological functions. For example, progestins are associated with the incidence and progression of breast cancer, whereas glucocorticoids are growth suppressive in mammary cancer cells. To understand the mechanisms that engender biological specificity, it is necessary to identify genes that are differentially regulated by the two receptors. Here we employ Affymetrix oligonucleotide arrays to compare glucocorticoid- and progestin-regulated gene expression in a human breast cancer cell line. This global analysis reveals that the two hormones regulate overlapping but distinct sets of genes, including

31 genes that are differentially regulated. Surprisingly, the set of differentially regulated genes was almost as large as the set of genes regulated by both hormones. Examination of the set of differentially regulated genes suggests mechanisms behind the distinct growth effects of the two hormones in breast cancer. The differential regulation of four genes representing different regulatory patterns was confirmed by RT-PCR and Northern blot analyses. Treatment with cycloheximide or RU486 indicates that the regulation is a primary, receptor-mediated event. Detailed analyses of genes identified in these studies will furnish a mechanistic understanding of differential regulation by glucocorticoids and progestins. (*Molecular Endocrinology* 16: 1204-1214, 2002)

**T**RANSSCRIPTION FACTORS ARE grouped into families based on their most conserved domain, the DNA-binding domain. In many cases, family members share similar, if not indistinguishable, DNA sequence recognition properties. Thus, a fundamental question in molecular biology concerns the extent to which related factors are functionally redundant in a given cell or tissue and the mechanisms by which factor-specific gene regulation is accomplished.

GR and PR are closely related members of the steroid receptor family of transcription factors (1). They share many similar structural and functional characteristics, including DNA sequence recognition specificity (2-6). The two receptors associate with a similar complex of molecular chaperones in the absence of hormone (7) and with a similar set of coactivators in the presence of hormone (8, 9). Despite the similarity of the two receptors, the cognate hormones display a very distinct spectrum of physiological actions. Classic actions of glucocorticoids include regulation of metabolism, inhibition of inflammation and the immune system, and suppression of bone formation (10). The major physiological role of progestins in the mammal are to establish and maintain pregnancy; to pro-

mote lobular-alveolar development in the mammary gland, and to suppress milk protein synthesis before parturition (11). Even in tissues that express both GR and PR, these two hormones may exert opposite biological actions. For example, in bone, glucocorticoids stimulate bone resorption (12-14), whereas progestins prevent bone loss (15, 16). In mammary gland, glucocorticoids promote milk protein synthesis and lactation (17-19), whereas progestins inhibit milk production and secretion (11). Furthermore, there is an association of progestins with the incidence and progression of breast cancer (20), whereas glucocorticoids are growth suppressive in mammary cancer cells (21, 22).

How can two receptors with such remarkable similarity mediate such dramatically different biological functions? Only a handful of cellular promoters regulated by GR or PR have been identified. Many of the studies on the mechanisms of GR and PR function have used the mouse mammary tumor virus promoter. This promoter is induced by both steroids under most circumstances, although chromatin environment may differentially influence mouse mammary tumor virus induction by the two hormones by mechanisms as yet poorly understood (23). Understanding the basis of the distinct physiology of glucocorticoids and progestins is severely limited by the paucity of genes and promoters identified to be differentially regulated by the two receptors. In this study, we have performed a global analysis of the gene regulation by glucocorticoids and

Abbreviations: Dex, Dexamethasone; DNase, deoxyribonuclease; 11 $\beta$ -HSD2, 11 $\beta$ -hydroxysteroid dehydrogenase type 2; INHBB, inhibin  $\beta$ B; NaGalase, N-acetylgalactosaminidase; PLZF, promyelocytic leukemia zinc finger protein; RNase, ribonuclease; VDUP1, vitamin D<sub>3</sub> up-regulated protein 1; VIP, vasoactive intestinal polypeptide.

progestins in a human breast cancer cell line using Affymetrix oligonucleotide microarrays. The results demonstrate that, in addition to genes regulated by both receptors, there is a set of genes that are differentially regulated. The systematic identification of such genes reveals potential avenues of differential regulation of cell growth by the two hormones and opens a new avenue for future studies on the molecular mechanisms underlying hormone-specific gene regulation.

## RESULTS AND DISCUSSION

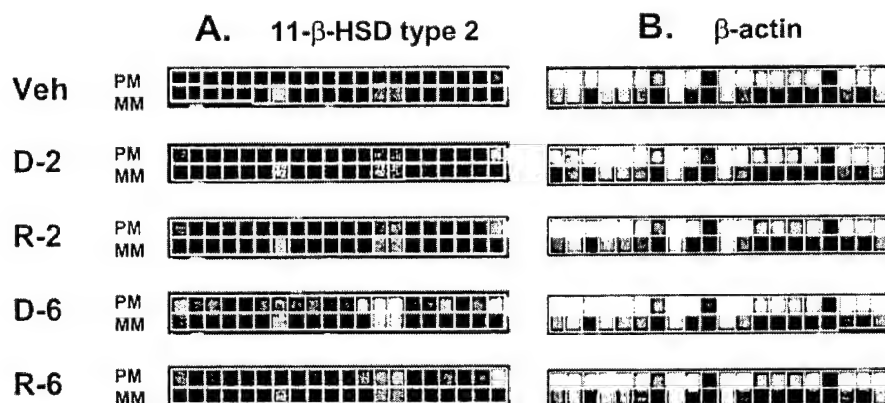
### Overlapping but Distinct Gene Regulation Profiles by Glucocorticoids and Progestins

To understand the basis of distinct actions of glucocorticoids and progestins in a tissue such as the mammary gland in which both receptors are expressed, and especially to identify genes that are differentially regulated by the two hormones, we performed microarray analysis of gene expression in the human breast cancer cell line T47D/A1-2. T47D/A1-2 cells express comparable levels of both GR and PR (24). Total RNA was isolated from cells treated with vehicle, dexamethasone (Dex, 100 nM), or R5020 (10 nM) for 2 h or 6 h. Probes generated from this RNA were hybridized to Affymetrix HuGeneFL arrays to analyze expression of 5,600 full-length human genes.

In each single array hybridized with cRNAs from T47D/A1-2 cells, 30–40% of the genes exhibit detectable expression. Figure 1 shows the fluorescent images of the probe sets in the arrays hybridized to cRNAs for 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) and  $\beta$ -actin. 11 $\beta$ -HSD2 has been shown

to be inducible by both glucocorticoids and progestins (25) and served as a positive control for the array analysis. As shown in Fig. 1A and Table 1, both Dex and R5020 treatment led to a dramatic increase in gene-specific hybridization. As an RNA loading control,  $\beta$ -actin hybridization signals did not change after either hormone treatment (Fig. 1B and Table 3).

Of 5,600 genes analyzed, 70 were up-regulated by glucocorticoids by more than 3-fold at either 2 h or 6 h treatment according to the criteria described in *Materials and Methods*, and another 33 were down-regulated by more than 3-fold. The number of genes that are up-regulated and down-regulated by progestins by more than 3-fold are 47 and 34, respectively. The majority of the genes (>90%) identified have not been previously described to be regulated by either glucocorticoids or progestins and therefore represent novel hormone-regulated targets. Among these genes, 25 were up-regulated by both hormones (Table 1), and 12 were down-regulated by both hormones by more than 3-fold (Table 2). Of particular interest for this work, 31 genes have been identified to be differentially regulated by the two hormones by more than 3-fold (Table 3). These results demonstrate that glucocorticoids and progestins regulate overlapping but distinct sets of genes. In light of the paucity of genes described to be differentially regulated by the two hormones, it was surprising that the number of genes differentially regulated by more than 3-fold, 31, approached the number regulated by both by more than 3-fold, 37. These represented about 1.5–2% of the genes whose expression could be detected. Novel hormone-regulated targets identified in this study will enhance our understanding of the role of these two hormones as both physiological regulators and phar-



**Fig. 1.** Transcript Monitoring by Hybridization to Oligonucleotide Arrays

The figure shows the fluorescent images of the probe sets hybridized to the cRNA for 11 $\beta$ -HSD2 (A) and  $\beta$ -actin (B). Each gene is detected by 20 vertical pairs of 25-mer oligos. The *top* oligo is a perfect match (PM), and the *bottom* oligo has a mismatch (MM) at the central position and serves as an internal control for hybridization specificity. The average difference in hybridization intensity between the PM and MM oligos is used to determine transcript level. Different oligos hybridize at different efficiency depending on sequence composition. The use of 20 pairs increases the accuracy of the measurement. A, Array results for a hormone-regulated gene. Expression of 11 $\beta$ -HSD2 is induced by both Dex and R5020 at 2 and 6 h. B, Array results for a constitutive gene. Expression of  $\beta$ -actin is unchanged with either hormone treatment, indicating equal RNA loading. Veh, Vehicle; D-2 and D-6, Dex (100 nM) for 2 h and 6 h; R-2 and R-6, R5020 (10 nM) for 2 h and 6 h.

**Table 1.** Genes Up-Regulated by both Dex and R5020 by More than 3-Fold

Probe Set	Description	Fold Change			
		D2:V	D6:V	R2:V	R6:V
D16227_at	BDP-1, recoverin family, calcium-binding	1.4	8.3	-2.5	5.7
D38037_at	FK506-binding protein 12-kDa homolog	1.6	9.2	4.2	5.6
D86956_at	KIAA0201 gene	1.8	3.5	2	3.1
HG4310-HT4580	Cellular retinol binding protein Li	3.6	10.7	1.5	7.3
M14218_at	Argininosuccinate lyase, arginine metabolism	2.2	3.9	3	2
M83667_rna1_s	NF-IL-6- $\beta$ protein, C/EBP family	4.1	8.7	3.6	7.5
U26726_at	11- $\beta$ -Hydroxysteroid dehydrogenase type 2	7.4	27.1	6.9	21
U36922_at	Fork head domain protein, transcription factor	1.5	4.9	3.1	3.2
U42031_at	54 kDa immunophilin FKBP54	1.7	6.3	1.5	3.8
U54999_at	LGN protein, inhibit G $\alpha$ i2	2.1	4.2	1.9	5.1
U62015_at	Cyr61, growth factor-inducible gene	1.5	3.4	1.9	5.1
U72342_at	Platelet activating factor acetylhydrolase	3.5	3.5	3.3	2.4
U73524_at	Putative ATP/GTP-binding protein	5.7	6.4	4.3	4.2
U77456_at	Nucleosome assembly protein 2	3.8	4.3	4	7.4
U81554_at	CaM Kinase Isoform II	6.3	5.4	5.4	4.2
U81556_at	Hypothetical protein A4	5.1	3.5	4.7	4.8
U83461_at	Putative copper uptake protein (hCTR2)	2.7	3.2	2.8	3.9
U85267_at	Down syndrome candidate region 1 (DSCR1)	1.6	4.7	2.4	7.7
U85611_at	DNA-PK interaction protein (KIP)	3.8	3.9	3.7	3.6
U90426_at	Nuclear RNA helicase	3.5	3.3	3.3	2.3
U90919_at	Clones 23667 and 23775 zinc finger protein	2.4	3	2.3	3.1
U91316_at	acyl-CoA thioester hydrolase	5.5	5.8	3.3	4.2
U91327_at	12p15 BAC clone CIT987SK-99D8	5.4	4.3	3.7	3.2
X60673_rna1	Adenylate kinase 3, nucleotide metabolism	2.9	3.2	3.3	3.2
Z19002 at	PLZF, transcription repressor	7.3	14.4	2.9	4.5

V, Vehicle; D2, D6, dexamethasone 2 h, 6 h; R2, R6, R5020 2 h, 6 h.

**Table 2.** Genes Down-Regulated by both Dex and R5020 by More than 3-Fold

Probe Set	Description	Fold Change			
		D2:V	D6:V	R2:V	R6:V
AB000584_at	Novel TGF- $\beta$ superfamily protein	-1.8	-20.7	-1.6	-20.6
D14695_at	KIAA0025 gene	1.1	-3.5	1.3	-3.5
HG2724-HT2820	Oncogene Tls/Chop, liposarcomas	-1.8	-14.8	-1.5	-21
L07615_at	Neuropeptide Y receptor Y1, G protein-coupled	-1.3	-3.5	-1.9	-4.4
L37347_at	Integral membrane protein, iron uptake	-2.9	-3	-2.6	-3.2
L37882_at	Frizzled gene, receptor for Wnt	-2.4	-6.7	-4.6	-6.8
M88461_s_at	Neuropeptide Y receptor YY, G protein-coupled	-2.2	-3.2	-2.6	-3.2
U07225_at	P2U nucleotide receptor	-1.7	-4.7	-1.7	-4.3
U07358_at	Protein kinase (zpk), a leucine zipper domain	-1.7	-3.7	-2.1	-3.2
U22376_cds2_s	c-myb, transcription factor, protooncogene	-1.5	-5	-3.3	-3
U39840_at	Hepatocyte nuclear factor-3 $\alpha$	-1.2	-3.3	-2.1	-3.3
X51630 at	Wilms tumor WT1, tumor suppressor	-1.2	-3.2	-1.3	-3.2

V, Vehicle; D2, D6, dexamethasone 2 h, 6 h; R2, R6, R5020 2 h, 6 h.

macological agents. (Please refer to the 10 Tables published as supplemental data on The Endocrine Society's Journals Online web site, <http://mend.endojournals.org/>.)

#### Confirmation of Differential Regulation with RT-PCR and Northern Blot Analyses

Four differentially regulated genes identified by the array analysis as representing different patterns of regulation were selected for further analysis. The first of these genes, G0S8 (also known as RGS2, regulator of G protein signaling 2) is specifically induced by Dex

but not by R5020 (Table 3). G0S8/RGS2 encodes a basic helix-loop-helix phosphoprotein (26) and specifically inhibits the function of Gq $\alpha$  as a GTPase activating protein (27). Knockout mice show reduced T cell proliferation and antiviral immunity, increased anxiety responses, and decreased male aggression (28). The selective glucocorticoid induction was confirmed by both semiquantitative RT-PCR and Northern blot (Fig. 2A). Quantitation of the Northern blot showed that Dex treatment led to a more than 20-fold induction, whereas R5020 treatment had minimal effect. The hormone-specific induction of G0S8/RGS2 suggests a selective coupling between glucocorticoid and G pro-

**Table 3.** Genes Differentially Regulated by Dex and R5020 by More than 3-Fold

Probe Set	Description	Diff. <sup>a</sup> D:R	Fold Change			
			D2:V	D6:V	R2:V	R6:V
L13391_at	G0S8, Gq $\alpha$ inhibitor	15.4	8.5	14.3	2.9	-1
Z19002_at	PLZF, transcription repressor	9.6	7.3	14.4	2.9	4.5
M38258_at	RAR $\gamma$ 1	6.5	-1.6	-1.2	-1.6	-7.3
U75272_s_at	Gastricsin, protein degradation	6.3	4	8.4	-1	1.3
S73591_at	VDUP1, inhibit thioredoxin and growth	5.1	1.2	1.1	-2.3	-4.5
U37546_s_at	IAP homolog C	4.6	1.6	4.7	2.1	-1.2
D80010_at	KIAA0188 gene	4.5	2.1	3.4	1.4	-1.9
HG3494-HT3688	Nuclear factor Nf- $\kappa$ B	4.2	-1.1	1.4	-1.2	-3.1
M77140_at	Pro-galanin, a neuropeptide	4.1	1.2	5.9	1.6	1.4
U15932_at	Dual-specificity protein phosphatase	4	1.2	-1	-3.4	-2.1
X77777_s_at	VIP receptor-related protein	4	3.8	10.7	-1.1	1.7
M33317_f_at	Cytochrome P450IIA4 (CYP2A4)	3.9	4.2	2.8	1.6	2.9
HG4310-HT4580	Cellular retinol binding protein Li	3.8	3.6	10.7	1.5	7.3
U63455_at	Sulfonylurea receptor	3.5	1.5	3.6	1.3	1
X17059_s_at	Arylamine <i>N</i> -acetyltransferase	3.4	3	2.8	1.7	1.2
X51956_rna1_at	ENO2, neuron specific enolase	3.2	1.4	-1.6	-1.1	-4.6
M29874_s_at	Cytochrome P450-IIB (hIIB1)	-3	-3.8	-3.7	-1.6	-2
HG110-HT110_s	Nuclear ribonucleoprotein A/B	-3.4	-4.8	1.1	-1.4	1.3
L13210_at	Mac-2 binding protein, tumor antigen	-3.4	1.1	1.3	3.7	-3
J03474_at	Serum amyloid A, acute phase	-3.5	1.4	1.6	3.5	1.3
M62783_at	$\alpha$ - <i>N</i> -Acetylgalactosaminidase	-3.9	-3.6	-1.2	1.1	-1
U34605_at	RA-, interferon-inducible	-3.9	-3.7	-2	-1	-1.7
U52513_at	RA-induced gene G	-4	-1.9	-4.4	3.1	-4.7
M31682_at	Testicular inhibin $\beta$ -B-subunit	-4.5	2	1.9	4.9	8.4
M80359_at	Protein p78	-5	-3.4	1.1	1.9	1.4
U23070_at	Putative transmembrane protein	-5	1	-4.2	1.9	1.2
X51441_at	Serum amyloid A (SAA), acute phase	-5.1	-1.5	1.1	4.1	1.3
U48807_at	MAPK phosphatase 2	-5.7	1.3	-3.6	1.1	1.6
U77735_at	Pim-2 protooncogene, Ser/Thr kinase	-6.2	1.5	1.1	3	5.3
S81914_at	IEX-1/Dif-2, growth factors inducible	-7.3	-4.8	-12	-2.4	-1.6
M74089_at	TB1 gene, FAP and colorectal cancer	-10.5	-8	1.2	1.3	1.2
Controls						
X00351_f_at	$\beta$ -Actin	-1.4	1	-1.3	-1.1	1.1
U37689_at	RNA polymerase II subunit	1.2	1.1	-1.1	-1.1	1

<sup>a</sup> Fold difference was calculated using GeneChip algorithms, not a direct ratio of fold changes. The higher value of the two timepoints is shown. V, Vehicle; D2, D6, dexamethasone 2 h, 6 h; R2, R6, R5020 2 h, 6 h.

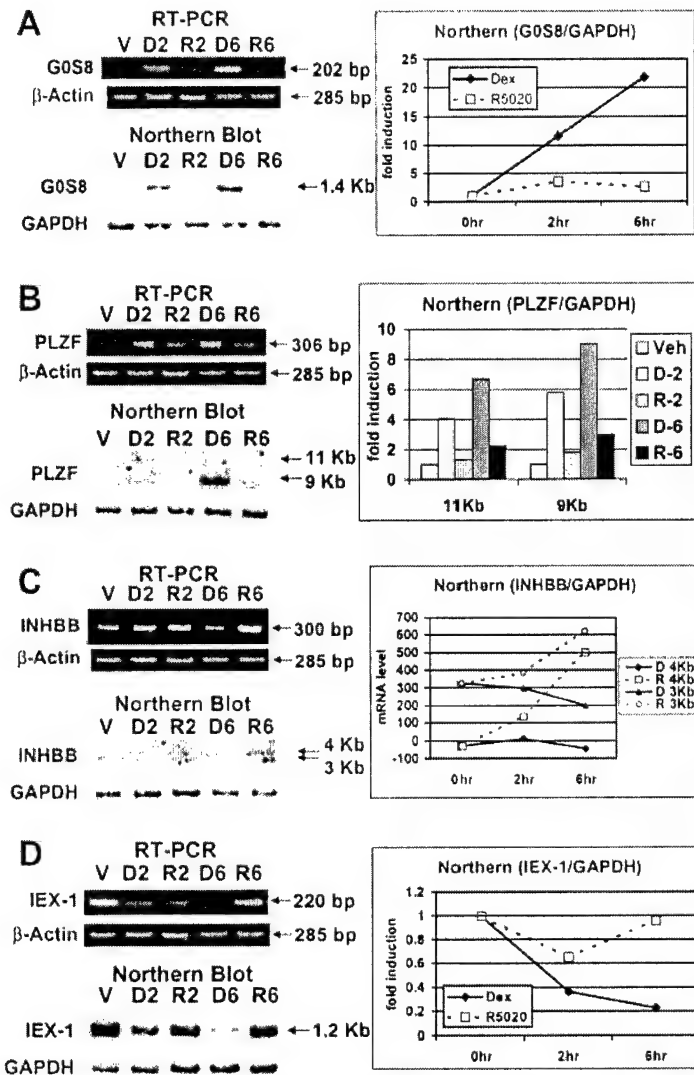
tein signaling. Previous studies have shown that glucocorticoids suppress the growth of Con8 rat mammary tumor cells by inducing a G1/G0 cell cycle arrest (21). Interestingly, G0S8/RGS2 has been shown to be induced in growth-arrested cells and to promote adipocyte differentiation (29). The induction of G0S8/RGS2 may also play a role in the growth-suppressive effect of glucocorticoids in mammary carcinoma cells.

The second gene analyzed, the promyelocytic leukemia zinc finger protein (PLZF), was originally identified as the fusion partner of the RAR $\alpha$  gene in a variant chromosomal translocation in acute promyelocytic leukemia (30). It is a transcription repressor with a kruppel-like zinc finger domain and a BTB/POZ domain (30, 31). PLZF represses transcription by recruiting a histone deacetylase through the silencing mediator of retinoid and thyroid hormone receptor-Sin3-histone deacetylase corepressor complex (32). Like G0S8/RGS2, RT-PCR and Northern blot analyses confirm that PLZF is strongly induced by Dex (Table 3 and Fig. 2B) but unlike G0S8/RGS2, PLZF is also

weakly induced by R5020. In addition, Northern blot showed that PLZF is expressed as two transcripts, 11 kb and 9 kb. Both transcripts are induced 3-fold higher by Dex than by R5020. PLZF has been shown to suppress the growth of myeloid cells by inducing G0/G1 arrest and apoptosis, partly through the binding and repression of the cyclin A2 promoter (33, 34). It is likely that the growth-suppressive effect of glucocorticoids in mammary carcinoma cells is also mediated, in part, through the robust induction of PLZF.

In contrast to PLZF and G0S8/RGS2, the array analysis suggests that the gene for the  $\beta$ B-subunit of inhibins and activins (INHBB for inhibin  $\beta$ B) is specifically induced by progestins but not glucocorticoids (Table 3). Inhibins and activins are members of the TGF $\beta$  superfamily, which are potent mediators of proliferation or antiproliferation and differentiation in different cell types. RT-PCR and Northern blot confirmed the array result (Fig. 2C). Northern blot showed that there are two transcripts for INHBB as seen previously for rat INHBB (35). R5020 treatment induced the ex-





**Fig. 2.** Expression Analyses of Selected, Differentially Regulated Genes

T47D/A1-2 cells were treated with vehicle (V or Veh), Dex (D, 100 nM), or R5020 (R, 10 nM) for 2 h (D2 or R2) or 6 h (D6 or R6). Total RNA was isolated and used for semiquantitative RT-PCR and Northern blot analyses. Quantitation of the Northern blot is shown on the right in each panel. A, G0S8, a gene specifically induced by glucocorticoids; B, PLZF, a gene preferentially induced by glucocorticoids; C, INHBB, a gene specifically induced by progestins; D, IEX-1, a gene preferentially down-regulated by glucocorticoids.

pression of both transcripts, whereas Dex treatment had no effect on the 4-kb transcript and slightly down-regulated the 3-kb transcript. Progesterone is a mammary morphogenesis essential for lobuloalveolar morphogenesis (36). Interestingly, female mice in which both alleles for INHBB have been deleted have lactation failure due to retarded ductal elongation and alveolar morphogenesis during puberty, pregnancy, and parturition (37). Our results suggest that the mammary effect of progesterone may be mediated, in part, by the specific induction of INHBB.

Unlike the previous three genes, the differential regulation of the IEX-1/Dif-2 gene involves a hormone-specific down-regulation. Northern blot demonstrated

that Dex treatment led to a dramatic 80% down-regulation by 6 h, whereas R5020 treatment resulted in a weak, transient down-regulation with expression returning to control levels by 6 h (Fig. 2D). IEX-1 was identified as a radiation-inducible immediate-early gene in human squamous carcinoma cells (38). It is also known as Dif-2 and is down-regulated during monocyte differentiation (39). The IEX-1/Dif-2 gene is induced by multiple signals, many of which are associated with proliferation, e.g. lipopolysaccharide,  $C_2$ -ceramide, lysophosphatidylcholine, phorbol esters, serum, or growth factors (39, 40). The association of IEX-1/Dif-2 with proliferation suggests that the growth-suppressive effect of glucocorticoids in mammary car-



cinoma cells is mediated, in part, through the down-regulation of IEX-1/Dif-2. The connection between additional genes differentially regulated by glucocorticoids and progestins and differential actions on mammary cell proliferation are discussed further below.

Previous studies have shown that the Affymetrix oligo array technology produces highly reliable results, many of which have been confirmed by conventional approaches (41–43). In this study, for all four genes tested individually, there is excellent agreement between the microarray results and the Northern blot analyses. The analysis of the promoters of these genes will provide a mechanistic understanding for differential regulation. Preliminary studies have mapped the glucocorticoid-mediated inhibition of TNF $\alpha$ -induced IEX-1/Dif-2 promoter activity to a small proximal promoter region containing a nuclear factor- $\kappa$ B element and juxtaposed SP-1-CCAAT enhancer binding protein elements (Wan, Y., unpublished data). The mutually inhibitory actions of GR and nuclear factor- $\kappa$ B have been well documented (44).

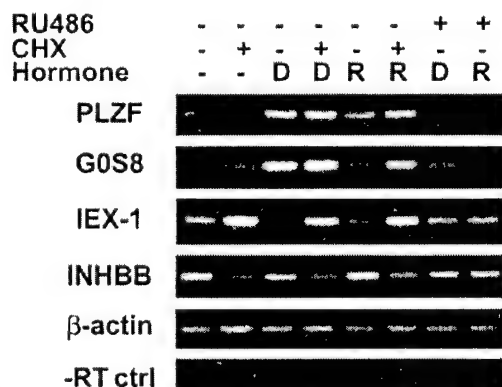
#### Hormone Regulation Is Direct and Receptor Mediated

The 2- and 6-h treatment times used for array analysis were chosen so that primary, receptor-mediated regulatory events, not secondary events, would be identified. To confirm that the differential regulation of the four genes above was mediated by the cognate receptors and direct, T47D/A1-2 cells were treated with protein synthesis inhibitor cycloheximide or the GR/PR antagonist RU486 along with Dex or R5020. Total RNA was isolated, and the expression of each gene was determined by semiquantitative RT-PCR (Fig. 3). Although cycloheximide itself can have an effect on mRNA levels, the hormone regulation pattern is maintained after cycloheximide treatment for all four genes, indicating that the hormone regulation is a direct effect

that does not require *de novo* protein synthesis. In contrast, the hormone regulation is completely abolished after RU486 treatment, indicating that hormonal regulation is mediated through GR and/or PR.

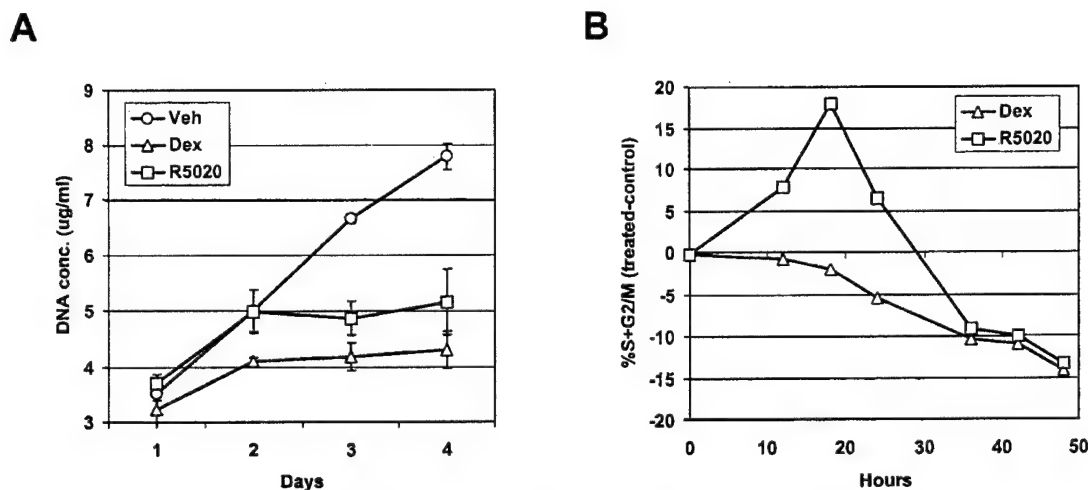
#### Differential Gene Regulation by Glucocorticoids and Progestins Mediate Hormone-Specific Effects

Progestins are associated with the incidence and progression of breast cancer (20). Recent work suggests that progestins may prime mammary cells to respond to growth factors (45). In contrast, glucocorticoids are growth suppressive in mammary cancer cells (21, 22). To test whether glucocorticoids and progestins have different effects on the proliferation of T47D/A1-2 cells, we assessed cell growth by monitoring the total DNA content of hormone-treated cell populations (Fig. 4A). The results demonstrated that Dex treatment inhibited cell growth throughout the time course, whereas R5020 treatment initially stimulated cell growth and then became inhibitory. To examine further the effect of glucocorticoids and progestins on the cell cycle progression of T47D/A1-2 cells within the first 24–48 h after hormone treatment, we also performed flow cytometry of propidium iodide-stained cells (Fig. 4B). Typical of a nonsynchronous population of proliferating cells, approximately 30% of untreated cells are in S or G2/M phases. Dex treatment decreased the fraction of cells in S+G2/M throughout the time course. By 48 h, the fraction of cells in S+G2/M had declined from 30% to 13%, suggesting that Dex treatment led to a cell cycle arrest in G1. This supports the observation in a previous study that Dex suppresses the growth of Con8 rat mammary tumor cells by inducing a G1/G0 cell cycle arrest (21). In contrast, R5020 treatment increased the fraction of cells in S+G2/M initially. At the peak (around 18 h), almost half of the cell population was in S+G2/M, a 63% increase over control. Together with the initial increase in DNA content, this indicates that progestins are growth promoting in the early hours of the treatment. By 30–36 h after R5020 treatment, the fraction of cells in S+G2/M dropped below controls to a level similar to Dex-treated cells, indicating that progestins are growth suppressive in the late hours of the treatment. This biphasic effect of progestins on the cell cycle progression of T47D cells has been previously described (46, 47). The current hypothesis is that progestin treatment initially drives cells to go through the first cell cycle to a decision point at the G1/S boundary; secondly, it induces cellular changes that permit other factors to influence the ultimate proliferative or differentiative state of the cells (45). For example, only progestin-primed T47D cells become highly sensitive to the proliferative effects of epidermal growth factor (46). In summary, the T47D/A1-2 cell growth and cell cycle studies clearly demonstrated that during the first 24 h of hormone treatment, glucocorticoids are growth suppressive, whereas progestins are growth stimulat-



**Fig. 3. Hormone Regulation Is Receptor Dependent and Protein Synthesis Independent**

T47D/A1-2 cells were treated with Dex (D, 100 nM), R5020 (R, 10 nM), cycloheximide (CHX, 40  $\mu$ g/ml), or RU486 (100 nM) for 6 h. Total RNA was isolated and semiquantitative RT-PCR performed with gene-specific primers.



**Fig. 4.** Glucocorticoids and Progestins Mediate Different Effects on the Growth of the T47D/A1-2 Cells

A, T47D/A1-2 cells were treated with vehicle, Dex (100 nM), or R5020 (10 nM) for the indicated number of days. Cell proliferation was determined by measuring DNA content in each well using the Hoechst DNA assay. The experiment shown is representative of three independent experiments each done in triplicate  $\pm$  SE. B, T47D/A1-2 cells were treated with vehicle, Dex (100 nM), or R5020 (10 nM) for the indicated number of hours. The fraction of cells in S+G2/M was measured by flow cytometry. The ordinate indicates the difference between the fraction of cells in S+G2/M in the hormone-treated sets and the fraction of cells in S+G2/M in the vehicle-treated controls. In this study, approximately 25–35% of control cells were in S+G2/M.

ing. The early time points (2 h and 6 h) used in our microarray analysis allowed us to assess the early molecular changes that may account for the differential growth effect of the two hormones on the T47D/A1-2 cells.

From the examination of the differentially regulated genes identified, a pattern emerged that gives insight into the differential effects of the two hormones on cell proliferation. A number of the genes that are preferentially induced by glucocorticoids or suppressed by progestins are growth suppressive (Table 3, top). G0S8/RGS2 and PLZF have been discussed above. Also of note are genes preferentially suppressed by R5020, including RAR $\gamma$ 1 and vitamin D $_3$  up-regulated protein 1 (VDUP1). RAR $\gamma$  inhibits proliferation and activates apoptosis in breast cancer cells (48, 49). VDUP1 suppresses cell proliferation by inhibiting the reducing potential of the disulfide reducing protein thioredoxin and down-regulating thioredoxin expression (50–52). Expression of VDUP1 is down-regulated in chemically induced rat mammary tumors (53). Thus, in mammary cancer cells, the growth-suppressive effect of glucocorticoids may be mediated through the induction of G0S8/RGS2 and PLZF, whereas the growth-promoting effect of progestins may be mediated through the down-regulation of RAR $\gamma$  and VDUP1.

In contrast, many of the genes that are preferentially induced by progestins or suppressed by glucocorticoids are potentially proliferation-related (Table 3, bottom). Mac-2 Binding Protein/90K is a tumor-derived antigen and is expressed at elevated levels in the serum of patients with breast cancer and other types of cancer (54).  $\alpha$ -N-Acetylgalactosaminidase (NaGa-

lase) is an extracellular matrix-degrading enzyme that is produced exclusively by cancer cells (55). The NaGalase levels in mice bearing squamous cell carcinoma increased with time of tumor growth and were directly proportional to tumor burden (56). INHBB promotes mammary gland ductal elongation and alveolar morphogenesis (37). MAPK phosphatase 2 can be induced by growth factors and is up-regulated in cells transformed by v-Jun or mutated K-ras (57, 58). Pim-2 is a protooncogene that induces lymphoid tumors synergistically with c-myc in mice (59, 60). IEX-1/Dif-2 gene can be induced by serum or growth factors (40) and is expressed in proliferating monocytes but significantly down-regulated during differentiation (39). In mammary cancer cells, the growth-suppressive effect of glucocorticoids may be mediated through the down-regulation of NaGalase, MAPK phosphatase 2, and IEX-1/Dif-2, whereas the growth-promoting effect of progestins may be mediated through the induction of Mac-2 BP/90K, INHBB, and Pim-2 h.

In addition to the effects on mammary cancer cell growth, glucocorticoids promote milk protein synthesis and lactation (17–19), whereas progestins inhibit milk production and secretion (11). Vasoactive intestinal polypeptide (VIP) receptor-related protein is the long isoform of VIP receptor. VIP has been shown to be a physiological mediator of PRL release in the rat (61). It is likely that glucocorticoid-specific stimulation of lactation is, in part, mediated by glucocorticoid-specific induction of VIP receptors (Table 3).

In summary, the genes identified to be differentially regulated by glucocorticoids and progestins provide potential mechanisms through which the two hormones exert different or opposite biological effects.

With a better understanding of the functionally uncharacterized genes identified here, more potential mechanisms will emerge. Future studies exploring these potential mechanisms will assist in developing tumor markers and therapeutic agents for cancer. In addition, genes identified in this study can be used as model systems to investigate the molecular mechanisms underlying differential gene regulation by glucocorticoids and progestins. The fruits of these investigations will enhance our appreciation of the larger question of how related transcription factors mediate distinct, even opposing, biological actions.

## MATERIALS AND METHODS

### Cell Culture and RNA Extraction

The T47D/A1-2 cell line was maintained in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% FBS (HyClone Laboratories, Inc., Logan, UT), 10 mM HEPES, nonessential amino acids, and 200  $\mu$ g/ml of G418. For RNA preparations, cells were grown until 80% confluence and fed with fresh medium the day before treatment and RNA extraction. Steroids and inhibitors were used at the following concentration: dexamethasone (100 nM), R5020 (10 nM), RU486 (100 nM), cycloheximide (40  $\mu$ g/ml). RNA was prepared using a Purescript RNA isolation kit (Gentra Systems, Minneapolis, MN). RNA was treated with ribonuclease (RNase)-free deoxyribonuclease I (DNaseI) (Ambion, Inc., Austin, TX) to remove all trace of genomic DNA, and the DNaseI was inactivated by 20 mM EDTA and heating at 75 C for 15 min. For preparing labeled cRNA for array hybridization, total RNA was also extracted with phenol/chloroform (Ambion, Inc.) and precipitated with ethanol.

### Preparation of Labeled cRNA and Array Hybridization

All procedures were performed according to the instructions from Affymetrix (Santa Clara, CA). Total RNA (10  $\mu$ g) was converted into double-stranded cDNA by using an oligo-dT primer with a T7 promoter at the 5'-end and the SuperScript Choice system for cDNA synthesis (Life Technologies, Inc.). Double-stranded cDNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 12  $\mu$ l RNase-free  $\text{dH}_2\text{O}$ . Half of the cDNA was used for *in vitro* transcription with a T7 RNA polymerase Megascript system (Ambion, Inc.) in the presence of biotinylated UTP and CTP (Enzo, Farmingdale, NY). The labeled cRNA was purified with RNeasy columns (QIAGEN, Chatsworth, CA), fragmented, and used to make up the hybridization cocktail containing control oligonucleotide B2 and four control bacterial and phage cRNAs (BioB, BioC, BioD, cre).

An aliquot of each sample was first hybridized to an Affymetrix Test 2 Array to determine sample quality according to manufacturer's criteria. All samples passed the test and were hybridized to a set of five Affymetrix GeneChip HuGeneFL Arrays, each carrying probes for about 5600 full-length human genes. After washing and staining, the arrays were scanned using a laser scanner controlled by the GeneChip 3.3 software (Affymetrix). To amplify the staining signal so that weakly expressed genes can also be detected, the cartridges were subjected to antibody amplification, second staining, and scanning.

### Data Analysis and Criteria for Selecting Regulated Genes

The scanned data were analyzed using GeneChip Expression Analysis Software (version 3.3, Affymetrix). To correct for minor differences in overall chip fluorescence, intensity values were scaled to 2500 so that the overall fluorescence intensity of each chip was equivalent (62).

It has been established that a change of 2-fold or greater is both significant and accurate using oligonucleotide array technology (63). In this study, a more stringent cut-off, a change of at least 3-fold at either of the two treatment time points, was used for selecting genes that are hormonally regulated. A 3-fold or greater change is deemed valid only when at least one sample in the comparison pair is scored as "present" and the change is also scored as "increase" or "decrease" by the GeneChip analysis software. The criteria for selecting genes that are differentially regulated by two hormones are: 1) a fold difference of at least 3 when comparing a Dex-treated sample with a R5020-treated sample at either time point; 2) a 3-fold or greater difference is deemed valid only when the change is scored as increase or decrease; 3) at least one of the four comparison pairs between hormone-treated sample and vehicle-treated sample has a valid change of at least 3-fold according to the criteria above.

### Semiquantitative RT-PCR

DNaseI-treated total RNA was reverse transcribed into single-stranded cDNA using random primers and SuperScript II or Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). For semiquantitative PCR, the best cycle number for linear amplification of the cDNA using each gene-specific primer pair was determined by amplifying serially diluted cDNA templates. The cDNA from each experiment sample was PCR amplified at this cycle, and the products were run on agarose gel and visualized by ethidium bromide staining.

### Northern Blot Analyses

DNaseI-treated total RNA (30  $\mu$ g) was separated on formaldehyde agarose gel and transferred to Hybond N<sup>+</sup> nylon membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). cDNA probes for G0S8, PLZF, and IEX-1 represent the entire coding sequences and were generated by RT-PCR and subcloning into vectors. A cDNA probe for INHBB was isolated from plasmid pSP65 (provided by Genentech, Inc., South San Francisco, CA). cDNA probes were labeled with a RediPrime II kit (Pharmacia Biotech, Piscataway, NJ) and hybridized with the membrane at 42 C overnight. After washing, the blot was quantitated by PhosphorImager with the STORM 860 system (Molecular Dynamics, Inc., Sunnyvale, CA).

### Cell Growth Assay

T47D/A1-2 cells ( $5 \times 10^4$ ) were plated into triplicate wells in 12-well plates with 2 ml of serum-containing medium. After 20–24 h, the cells were treated with 1/1,000 volume of vehicle (95% ethanol), Dex (100 nM), or R5020 (10 nM). Cells were harvested every 24 h after hormone addition, and the DNA content in each well was measured using a Hoechst DNA assay as described previously (64). The culture medium was removed and 300  $\mu$ l of 0.5 M NaOH were added to each well and pipetted up and down. The 12-well plates were frozen immediately at  $-20$  C until all time points were collected. After thawing, samples were pipetted to dissolve DNA and were neutralized with an equal volume of 0.5 M HCl + 200 mM phosphate buffer. Two aliquots of each sample were mixed with equal volumes of Hoechst stain (0.2  $\mu$ g/ml Hoechst

33258 in phosphate buffer containing 4.6 M NaCl) and were incubated in the dark at room temperature for 45 min. The relative fluorescence of each sample was measured using a MicroFLUOR plate reader (Dynatech Corp., Chantilly, VA). Five known concentrations of calf thymus DNA were used to develop a standard curve from which relative fluorescence units could be converted to micrograms per milliliter DNA for each sample.

### Flow Cytometry

T47D/A1-2 cells ( $3 \times 10^5$ ) were plated into duplicate wells in six-well plates with 3 ml of serum-containing medium. After 28–30 h, the cells were treated with 1/10,000 volume of vehicle (95% ethanol), Dex (100 nM), or R5020 (10 nM). Cells were harvested at the start of treatment (0 h) and every 6 or 12 h after hormone addition with trypsin-EDTA. The cell suspension was pelleted, washed with cold PBS, and resuspended in 1 ml of Krishan's stain (65) containing propidium iodide and ribonuclease (RNase A). Samples were stained at 4°C for at least overnight before flow cytometry. For each sample, 10,000 cells were analyzed on an Epics 752 flow cytometer (Coulter Electronics, Hialeah, FL) using an incident beam on forward angle vs. 90° light scatter to eliminate cellular debris and doublets. Red fluorescence, corresponding to DNA, was collected through a 590-nm long pass filter, and histograms of DNA content vs. cell number were constructed. Cell cycle analyses of the DNA histograms were performed using the ModFit Analysis Program (Veritey Software House, Topsham, ME), which provides fits for the G0/G1, S, and G2/M fractions of the population. The S- and G2/M-phase fractions were combined into a single growth fraction. In Fig. 4, the ordinate shows the difference between the fraction of cells in S+G2/M in the hormone-treated sets and the fraction of cells in S+G2/M in the vehicle-treated controls.

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